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NOTE ON THE PREPARATION OF MANNOSE.

BY E. P. CLARK.

(From the Polarimetry Section, United States Bureau of Standards, Washington.)

A method for the preparation of mannose from ivory nut shavings has recently been described by Horton.[1] In his procedure the shavings are first treated with dilute sodium hydroxide solution, whereby gums and extractives are removed. This preliminary step is a great improvement over previous methods, as it gives a product of higher purity and eliminates the disadvantage of excessive foaming when the solution is concentrated. However, the mass of detail prescribed for subsequent steps offsets the advantage gained, and hence the method, as a whole, is no improvement over that of Hudson and Sawyer.[2]

The writer has on various occasions prepared mannose by a very simple process which, when applied to ivory nut shavings that have first been treated with sodium hydroxide, gives a yield that is considerably higher than either author has reported. This method is given below, as its simplicity and economy will appeal to workers who have to prepare this sugar.

Sifted ivory nut shavings are added to ten times their weight of boiling 1 per cent sodium hydroxide solution. The mixture is at once removed from the source of heat and stirred occasionally during $\frac{1}{2}$ hour. The shavings are then washed thoroughly with running water until neutral and clear, and dried.

500 gm. of the material, thus prepared, are thoroughly mixed with 500 gm. of 75 per cent sulfuric acid and allowed to stand until the next day. This mass is dissolved in water, making a volume of 5.5 liters, and boiled under a reflux for $2\frac{1}{2}$ hours. While the liquid is still boiling, it is neutralized with a thin paste of

[1] Horton, P. M., *J. Ind. and Eng. Chem.*, 1921, xiii, 1040.

[2] Hudson, C. S., and Sawyer, H. L., *J. Am. Chem. Soc.*, 1917, xxxix, 470.

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precipitated barium carbonate. The solution is at once filtered through a thin layer of active carbon placed on moistened filter paper in a Buchner funnel. The filtrate generally contains a little barium, probably in combination with organic acids. This is removed by adding a few cc. of dilute sulfuric acid until no further precipitate is formed. The barium sulfate is filtered off and the solution evaporated under reduced pressure to 87 to 88 per cent total solids.[3] An equal volume of glacial acetic acid is added and thoroughly mixed by warming and shaking. The syrup is seeded, placed in an ice box over night for crystallization to start, and it is then frozen with an ice-salt mixture. The frozen mass is placed in a refrigerator at or near 0°C. where it will thaw out slowly. After about a day the greater portion of the sugar will often have crystallized, but generally a week is required for complete crystallization. The yield is uniformly 42 to 45 per cent of the treated meal used.

[3] Horton directs that the final syrup shall be concentrated to 96 per cent total solids. It is difficult and tedious to get such a thick mixture into solution in acetic acid, and it is obviously impractical where 2 or more kilos of mannose are to be made at one time. A concentration of 87 to 88 per cent total solids is amply heavy, and offers no difficulty either in dissolving in the acid or in subsequent crystallization.

THE EFFECT OF LOSS OF CARBON DIOXIDE ON THE HYDROGEN ION CONCENTRATION OF URINE.

By E. K. MARSHALL, Jr.

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In the course of certain investigations which involved a study of the elimination of carbonates by the kidney, and the simultaneous determination of hydrogen ion concentrations in urine, it was noticed that under certain conditions the escape of carbon dioxide from the urine might have a decided influence on the hydrogen ion concentration. The importance of preventing the loss of carbon dioxide or of equilibrating to a definite tension of carbon dioxide in determining the hydrogen ion concentration of blood is now too well recognized to need comment. The same extreme precautions in regard to escape of carbon dioxide have also been found necessary in making determinations on spinal fluids. The marked discrepancies and much too low values in the earlier work due to neglect of proper precautions have been recently pointed out by Parsons and Shearer (1). No precautions have generally been taken in collecting urine for the determination of hydrogen ion concentration to prevent the loss of carbon dioxide. It has, apparently, been generally assumed that since in urine the buffer action is mainly due to the phosphates, the carbonates are too small in amount to play any considerable rôle. In blood plasma the bicarbonate-carbon dioxide is the main buffer system, the phosphates being present in only very small relative concentrations. However, a cursory examination of the carbon dioxide and phosphate concentrations in urine indicates that in the case of urines of low hydrogen ion concentration (as obtained after administration of alkali or an alkaline diet and after forced breathing) and also in the case of

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dilute urines obtained during diuresis the conditions may be very similar to those in blood plasma and loss of carbon dioxide would have an appreciable effect on the hydrogen ion concentration. For instance, a urine obtained after the administration of sodium bicarbonate by mouth, contained 270 volumes of carbon dioxide and 60 mg. of PO_4 per 100 cc. The relative rôle of the bicarbonate and phosphate as buffers is here about the same as of blood. The pH immediately after voiding was 7.6 but after shaking for a few minutes rose to 8.8. In case of acid concentrated urines as ordinarily obtained on a mixed diet without excessive fluid intake, the loss of carbon dioxide will not have an appreciable influence in lowering the hydrogen ion concentration.

Very few determinations of the carbon dioxide content of urine have been made except in isolated instances. The recent paper by Denis and Minot (2) contains the most data available upon the subject. However, it is evident that no special precautions were taken by these investigators to avoid loss of carbon dioxide during voiding. They state that "no change is demonstrable in the carbon dioxide content of either acid or alkaline urines, preserved with chloroform, and kept in well stoppered containers at room temperature for 24 hours." The data which are given to support this statement show that analyses were made 15 minutes after voiding and 24 hours later. How much loss took place in the first 15 minutes is not stated, but from our own results we know that it may have been considerable. A comparison of their data with that contained in this paper indicates that their results are considerably lower for the carbon dioxide content of urine.

The carbon dioxide was determined by means of the Van Slyke apparatus (3) using 0.5 to 5 cc. of urine depending on the concentration present. A double extraction was always made and the carbon dioxide was absorbed with alkali. The total carbon dioxide (free and combined) was then calculated from the volumes of gas obtained. The hydrogen ion concentrations were determined by means of Henderson and Palmer's method (4) with modifications in the choice of indicators and amounts of urine used. Frequently the determinations were made on the undiluted urine, and were occasionally made under oil to

prevent any possible escape of carbon dioxide. All the specimens were obtained from normal men. Urine was collected by having the subject void into a narrow cylinder with as little dropping of the stream through air as possible. A sample for the carbon dioxide was transferred to the apparatus in less than 1 minute. The hydrogen ion concentration was also determined at once. An estimate of the amount of the loss of carbon dioxide in using this procedure was obtained as follows: A sample of urine was collected without exposure to air by a method similar to that used by Fredericq (5) and another sample was obtained at the same time by voiding into a cylinder or by allowing the urine obtained without exposure to air to run through air. The carbon dioxide and hydrogen ion concentration were determined

TABLE I.

Experiment No.	Collected without exposure to air.		Collected with exposure to air.	
	vol. per cent CO₂	*pH*	*vol. per cent CO₂*	*pH*
1	8.06	6.10	7.48	6.15
2	14.4	6.55	13.5	
3	45.6	7.0	43.5	7.1
4	13.0	6.3	12.1	6.3
5	4.72	5.5	4.48	5.5
6	3.98		3.74	5.1

in each as quickly as possible. The data in Table I indicate that the loss by the method used is less than 10 per cent.

In Table II are given data on the carbon dioxide content of urines of different hydrogen ion concentrations. The results are slightly low, but are probably accurate within 10 per cent and are higher than those of Denis and Minot (2) for the different hydrogen ion concentrations. They show, as pointed out by Denis and Minot, that the carbon dioxide content varies inversely but not proportionately to the hydrogen ion concentration.

The magnitude of the error which may be caused by the escape of carbon dioxide from urine in the determination of the hydrogen ion concentration is shown in Table III. Urine samples were collected and the pH determined at once. A sample (5 to 10 cc.) was then poured into a 250 cc. Pyrex flask and shaken for 1 minute, and the pH again determined. This was judged to represent

roughly the agitation of samples which might take place where no precautions were taken. Determinations are also included of

TABLE II.

Number of observations.	pH	Volume per cent CO₂.	
		Range.	Average.
8	5.05–5.20	3.00–4.74	3.77
8	5.30–5.40	3.45–6.70	4.00
5	5.50–5.70	4.56–5.22	5.01
8	5.90–6.10	4.60–8.76	6.01
3	6.20–6.45	5.22–12.70	8.70
2	6.90–7.00	45.6–63.0	
1	7.50	264	

TABLE III.

Effect of Loss of Carbon Dioxide on pH of Urine.

Determinations of pH before and after shaking 1 minute.

Before.	After.	Before.	After.
5.10	5.15	6.80	7.60
5.50	5.50	6.90	7.55
5.50	5.55	7.25	8.10
5.60	5.60	7.25	8.40
5.50	5.90	7.30	8.10
5.90	6.10	7.30	8.15
5.90	6.40	7.45	8.50
6.30	6.50	7.50	8.30
6.45	6.65	7.80	8.60

Determination of pH before and after shaking 10 minutes.

Before.	After.	Before.	After.
5.25	5.30	6.90	8.50
5.50	5.70	7.20	8.80
5.90	6.60	7.30	8.20
5.95	6.25	7.50	8.40
5.95	6.45	7.55	8.90
6.30	6.60	7.70	8.80
6.45	6.75	7.80	9.00

samples shaken for 10 minutes. The more alkaline specimens (third and fourth columns of table) were obtained after the ingestion of 3 to 10 gm. of sodium bicarbonate.

The effect of loss of carbon dioxide on the hydrogen ion concentration is seen to be very slight in the case of acid urines. In some cases the effect is, however, quite pronounced and in these cases the urine was noticed to be very dilute. In a number of the cases recorded in Table III, it was found that 10 minutes shaking removed practically all of the carbon dioxide originally present (or the carbon dioxide tension was approximately that of atmospheric air). Change of reaction is prevented in a concentrated urine by the efficient concentration of the buffers present. In dilute urines the efficiency of the phosphates as buffers is decreased because it has been shown that water diuresis causes only a slight increase in the total amount of phosphate elimination, while the carbonate is markedly increased in absolute amount and generally in percentage (6). An examination of the neutral or alkaline urines obtained after the administration of sodium bicarbonate indicates that the error due to the escape of carbon dioxide is very great. Even after 10 minutes shaking, only a small part of the carbon dioxide is removed from the more alkaline urines. This is to be expected as it is well known that on passing atmospheric air through a dilute solution of sodium bicarbonate equilibrium is attained very quickly (7), while a concentrated bicarbonate solution (0.1 molar or stronger) requires a long passage of the air current to establish equilibrium with the carbon dioxide of the air (8). The final hydrogen ion concentration when equilibrium is established will depend, of course, on the original concentration of bicarbonate. In these urines of high bicarbonate content, the other buffers (phosphates, etc.) are in insufficient concentration to prevent change of reaction, when carbon dioxide is allowed to escape.

The values given in Table III for the initial pH of urine before shaking may be slightly high in the case of the alkaline urines owing to the escape of carbon dioxide during collection. Some experiments carried out on dogs illustrate this very well. The following is an example.

A dog of 6.3 kilos weight was anesthetized with paraldehyde, and the ureters cannulated. The cannulas were constructed so that the ends could be placed in narrow cylinders containing a layer of paraffin oil. Diuresis was produced by the intravenous injection of 10 cc. of 10 per cent sodium chloride solution. Specimens of urine were collected from each ureter in

such a way that the urine did not come in contact with air. These are designated R1 and L1. 15 cc. of 10 per cent sodium bicarbonate were then injected intravenously and specimens of urine collected (R2 and L2). 20 cc. of a 10 per cent sodium phosphate solution (pH 7.4) were then injected, and specimens collected as before (R3 and L3). Finally, a second injection of sodium bicarbonate was given and the urine from each kidney collected (R4 and L4). The hydrogen ion concentrations were determined at once, and estimations made of the total carbon dioxide present. A sample was withdrawn from under the oil with a pipette, allowed to flow into a flask, and after standing open for 15 minutes the pH was determined. Samples were then shaken in a flask for about 10 minutes. Table IV contains the data.

TABLE IV.

Specimen.	pH at once.	pH later.	pH after shaking.	CO₂	PO₄
				vol. per cent	*mg. per 100 cc.*
R1	5.6	6.2			
L1	5.6	6.1			56
R2	7.3	7.8	8.8	87.6	38
L2	7.3	7.8		118.0	50
R3	6.5	6.8	6.9	30.0	1,035
L3	6.5	6.8		27.3	1,120
R4	7.3	7.8	8.7	100.0	133
L4	7.3	7.8		98.5	122

The lowest hydrogen ion concentrations which have been reported for urine may be too low due to neglect of precautions to avoid the escape of carbon dioxide. Henderson and Palmer (9) found the limits of hydrogen ion concentration in human urines to be between pH 4.7 and 8.7. Their determinations were made apparently without any precautions to avoid loss of carbon dioxide as 24 hour collections were frequently used. 2 to 3 hours after giving 8 gm. of bicarbonate by mouth they frequently obtained urines of pH 8.7. In three normal individuals, doses of 5 to 15 gm. of sodium bicarbonate yielded urines of a maximum pH of 8.0 in one case, and usually no higher than 7.8. These urines were collected by voiding into a narrow cylinder as described, and hence may have lost some carbon dioxide during voiding. Urines of pH 8.7 and 9.27 have been reported for rabbits fed on carrots and oats (10), but may be due to escape of carbon dioxide. Urine was collected by catheter under oil from a rabbit fed on

lettuce, celery, and cabbage. It had a pH of 8.0, but when removed from under the layer of oil and shaken the pH rose to 9.2.

The intravenous injection of sodium carbonate into dogs in large amounts yielded samples of urine of only pH 8.0 when proper precautions were taken to prevent loss of carbon dioxide.

A dog of 10.2 kilos was anesthetized with paraldehyde, and the ureters were cannulated. A marked diuresis was produced by the injection of hypertonic sodium chloride intravenously. Urine was collected from the right ureteral cannula by connecting a 1 cc. Ostwald pipette filled with neutral distilled water, without admitting any air bubbles. The upper part of the stem of the pipette was filled with a solution of an indicator (bromocresolphthalein or phenolsulfonephthalein). The urine was allowed to displace the water. A sufficient amount of the indicator remained in the bulb to allow a comparison with solutions of known pH contained in similar pipettes. It required only 1 to 3 minutes to fill the pipette, so that several determinations could be made in a short time. Alternately with the collection of samples by means of the pipette, samples were collected by allowing urine to drop from the cannula into small test-tubes. The pH was determined on these at the close of each period of the experiment. Care was taken not to shake the test-tubes. In the first period of the experiment eight determinations by means of the pipettes gave pH values varying from 6.6 to 6.8 with an average of 6.74, while a similar number of determinations in the test-tubes gave 6.7 to 6.95 with an average of 6.83. In the second period 10 cc. of 10 per cent sodium carbonate were injected intravenously and determinations made as before. Ten samples collected with pipettes showed pH values from 7.4 to 7.6 with an average of 7.49 while eleven with the other method varied from 7.8 to 8.0 with an average value of 7.90. In the third period 45 cc. more of 10 per cent carbonate were injected. Of eight samples collected without exposure to air, one had a pH of 8.0, two of 7.9, and the remainder 7.8, while alternate samples collected with exposure to air gave one value of 8.2, five of 8.1, and two of 8.0. A sample of urine collected under oil from a cannula in the left ureter during the entire third period had a pH of 7.8, and contained 500 volumes per cent of total carbon dioxide (free and combined).

It is possible that a lower hydrogen ion concentration may be obtained in urine after the administration of much larger doses of sodium bicarbonate. But, since carbon dioxide is very diffusible it is probable that the free carbon dioxide or the tension of the gas in urine is the same as that in the renal tissue. It has been found that the acetone concentration in urine is the same as that in the blood plasma (11). If the tension of carbon dioxide varies in urine within narrow limits the maximum hydroxyl ion concentration is conditioned by the concentration of bicarbonate present. Davies, Haldane, and Kennaway (12), who admin-

istered to themselves very large amounts of sodium bicarbonate found a maximum concentration of bicarbonate in urine of about 0.22 molar, which was not exceeded if larger amounts of bicarbonate were taken. The concentration of bicarbonate in some of the urines which I have examined approached very near to this figure. Therefore, it is probable that the urine of man is never of an alkalinity higher than pH 8.0.

Strassburg (13) and Fredericq (5) have determined the carbon dioxide tension in urine and found it to vary from about 8 to 14 per cent of an atmosphere. This has been taken as an approximate measure of the tension of this gas in the renal tissues (14). The carbon dioxide tension in the blood is given by determining its tension in the alveolar air, but this varies considerably in different individuals and in the same individual under different conditions (15). Hasselbalch (16) has shown that a diet which produces an acid urine causes a low carbon dioxide tension of blood, while a diet which produces a less acid or alkaline urine causes a high carbon dioxide tension of blood. It is probable that the carbon dioxide tension of the urine will be found to bear a simple relation to that of the alveolar air. With this relation established, it would be an easy matter to equilibrate samples of urine to the proper tension, and determine the hydrogen ion concentration with great accuracy.

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STUDIES IN CARBOHYDRATE METABOLISM.

III. A STUDY OF URINARY SUGAR EXCRETION IN TWENTY-SIX INDIVIDUALS.

By ISAAC NEUWIRTH.

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No figures on the daily output of urinary sugar in normal individuals have been published since the work of Benedict, Osterberg, and Neuwirth appeared (1). As this earlier work was limited to the data obtained from two subjects, advantage has been taken of an opportunity to extend this study to a number of other normal individuals.

In the earlier work of Benedict, Osterberg, and Neuwirth, the original method of Benedict and Osterberg (2) was used. In the present investigation, the new method of these authors was employed (3). All urines were preserved with toluene during their collection. They were analyzed for reducing substances both before and after fermentation. Dextrose was always used as a check on the activity of the yeast. In all cases, qualitative examinations of the urines were made for sugar, albumin, and indican by means of the Benedict, heat-acetic acid, and Obermayer's tests, respectively.

The majority of the subjects were medical students.[1] They were interested in the problem and fully understood the significance of obtaining complete 24 hour specimens of the urine; wherever there was any doubt as to the completeness of the 24 hour specimen, the specimen was rejected. The work was

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planned so as to obtain two 24 hour specimens of urine from each individual. The subjects were requested to make no change in their routine of living. Records were kept of the food taken by each subject in these experiments, but the diet was not weighed. The diets are not reported here because it is questionable whether a record of the unweighed food intake would justify the use of the large amount of space necessary to reproduce these records. All subjects except Nos. 12, 16, and 26 were males.

Table I gives the findings for twenty-two normal persons. In Table II are recorded the results for four individuals—two of these are apparently normal, although the mother of each is diabetic; the urines of the other two subjects showed some abnormality; *i.e.*, in one case (Subject 25), the urine gave a slight qualitative test for sugar, in the other (Subject 26), albumin was present in the urine. Data as to weight and height are included because of their suggested relationship to the onset of diabetes (Joslin, 4).

It can very readily be seen from Table I that the quantity of sugar excreted is quite independent of volume of the urine, a conclusion reached by previous investigators (1, 5).

A study of figures and detailed diets in Table I reveals very plainly in some cases the marked influence of the diet on sugar excretion. For example, in the case of Subject 6, the total sugar dropped from 898 to 681 mg., coincident with a marked restriction in the intake of food on the 2nd day. Likewise, Subject 4 shows a marked increase in total sugar on the 2nd day (637 to 1,037 mg.), a result coincident with the ingestion of a very large bar of chocolate and other carbohydrate foods on the 2nd day.

Subject 20 shows an unusual relationship between the fermentable and non-fermentable sugars. The non-fermentable sugar in his case makes up over 80 per cent of the total sugar in each of the three urines reported, as compared with 51 to 75 per cent, the range found in all other normal cases here reported.

The figures reported for a boy of 13, Subject 22, are similar to those reported for the adult.

The figures in Table I are of special interest. Here are given the total reducing substance in amount and absolute percentage, the amount of reducing substance which was lost by fermentation and the amount and absolute percentage remaining after fermen-

TABLE I.

Subject.	Age.	Weight.	Height.	Date.	Volume of urine.	Before fermentation.		Fermented.	After fermentation.		Non-fermented.
		lbs.	inches	1921	cc.	per cent	mg.	mg.	per cent	mg.	per cent
1	29	127	61.5	Jan. 11	1,280	0.063	806.4	204.8	0.047	601.6	74.6
				" 12	1,190	0.069	821.1	249.9	0.048	571.2	69.6
2	21	130	66.5	" 12	1,315	0.082	1,078.3	447.1	0.048	631.2	58.5
				" 14*	1,120	0.103	1,153.6				
				" 28	1,060	0.101	1,070.6	487.6	0.055	583.0	54.4
3	21	210	73	" 24	3,100	0.042	1,302.0	403.0	0.029	899.0	69.0
				" 26	2,265	0.046	1,041.9	362.4	0.030	679.5	65.2
4	19	115	62	" 25	995	0.064	636.8	199.0	0.044	437.8	68.8
				" 27	910	0.114	1,037.4	400.4	0.070	637.0	61.4
5	22	158	65.5	" 19	1,370	0.076	1,041.2	438.4	0.044	602.8	57.9
				" 24	1,490	0.073	1,087.7	342.7	0.050	745.0	68.5
6	24	135	71	" 24	1,360	0.066	897.6	299.2	0.044	598.4	66.7
				" 26	1,840	0.037	680.8	184.0	0.027	496.8	73.0
7	25	155	69.5	" 27	705	0.109	768.5	359.6	0.058	408.9	53.2
				Feb. 2	1,275	0.072	918.0	446.2	0.037	471.8	51.4
8	25	170	70	Jan. 27	1,020	0.093	948.6	244.8	0.069	703.8	74.2
				Feb. 2	1,190	0.077	916.3	261.8	0.055	654.5	71.4
9	21	180	73.5	Jan. 27	1,280	0.089	1,139.2	371.2	0.060	768.0	67.4
				Feb. 3	1,240	0.090	1,116.0	409.2	0.057	706.8	63.3
10	23	140	71	Jan. 27	1,335	0.086	1,148.1	440.5	0.053	707.6	61.6
				Feb. 1	1,140	0.096	1,094.4	387.6	0.062	706.8	64.6
11	21	130	66.5	Jan. 28	1,470	0.072	1,058.4	411.6	0.044	646.8	61.1
				Feb. 7	895	0.099	886.1	420.7	0.052	465.4	52.5
12	22	110	59	" 14	1,008	0.079	795.6	362.5	0.043	433.1	54.4
				" 17	740	0.083	614.2	244.2	0.050	370.0	60.2

* These figures are not included in the average.

TABLE I—*Concluded*.

Subject.	Age.	Weight.	Height.	Date.	Volume of urine.	Before fermentation.		Fermented.	After fermentation.		Non-fermented.
		lbs.	inches	1921	cc.	per cent	mg.	mg.	per cent	mg.	per cent
13	20	120	63	Feb. 10	700	0.097	679.0	280.0	0.057	399.0	58.8
				" 16	770	0.112	862.4	423.5	0.057	438.9	50.9
14	20	130	66.5	Jan. 31	1,115	0.076	847.4	401.4	0.040	446.0	52.6
				Feb. 7	1,190	0.082	975.8	428.4	0.046	547.4	56.1
15	22	135	65	Jan. 31	1,210	0.073	883.3	375.1	0.042	508.2	57.5
				Feb. 3	1,050	0.093	976.5	462.0	0.049	514.5	52.7
16	19	125	64	" 1	760	0.100	760.0	349.6	0.054	410.4	54.0
				" 3	415	0.208	863.2	398.4	0.112	464.8	53.8
17	23	120	64	" 1	800	0.111	888.0	272.0	0.077	616.0	69.4
				" 3	800	0.124	992.0	384.0	0.076	608.0	61.3
18	19	135	66.5	" 8	1,560	0.064	998.4	358.8	0.041	639.6	64.1
				" 9	1,265	0.085	1,075.3	354.2	0.057	721.1	67.1
19	22	145	65.5	" 8	1,330	0.104	1,383.2	359.1	0.077	1,024.1	74.0
				" 10	905	0.110	995.5	298.6	0.077	696.9	70.0
20	20	145	70	" 8	820	0.104	852.8	139.4	0.087	713.4	83.7
				" 15	700	0.135	945.0	175.0	0.110	770.0	81.5
				Mar. 30	790	0.120	948.0	134.3	0.103	813.7	85.8
21	23	135	69.5	Feb. 9	1,250	0.086	1,075.0	300.0	0.062	775.0	72.1
				" 16	1,560	0.062	967.2	327.6	0.041	639.6	66.1
22	13	75	57.5	" 26	1,250	0.060	750.0	200.0	0.044	550.0	73.3
				Mar. 5	1,560	0.047	733.2	218.4	0.033	514.8	70.2
Maximum....................					3,100	0.208	1,383.2	487.6	0.112	1,024.1	85.8
Minimum....................					415	0.037	614.2	134.3	0.027	370.0	50.9
Average....................					1,183.4	0.079	941.2	333.7	0.051	607.5	64.5

tation. In the last column is given the proportion of non-fermentable reducing substance as percentage of the total reducing substance.

The total sugar output for 24 hours varies from 614 to 1,383 mg.; the fermentable reducing substance varies from 134 to 488 mg.; the non-fermentable reducing substance varies from 370 to 1,024 mg. The non-fermentable sugar amounts to from 51 to 86 per cent of the total sugar. The absolute percentage of sugar in the urine before fermentation varies from 0.037 to 0.208 per cent; after fermentation, it varies from 0.027 to 0.112 per cent.

TABLE II.

Subject.	Age.	Weight.	Height.	Date.	Volume of urine.	Before fermentation.		Fer-mented.	After fermenta-tion.		Non-fermented.
		lbs.	inches	1921	cc.	per cent	mg.	mg.	per cent	mg.	per cent
23*	35	135	67	Jan. 19	2,160	0.048	1,036.8	324.0	0.033	712.8	68.8
24*	21	150	64	" 11	2,300	0.063	1,449.0	667.0	0.034	782.0	54.0
				" 13	2,140	0.092	1,968.6	1,176.8	0.037	791.8	40.2
				" 27	1,405	0.077	1,081.9	491.8	0.042	590.1	54.5
				Feb. 24	1,800	0.055	990.0	522.0	0.026	468.0	47.3
25	20	125	65.5	Jan. 19†	1,400	0.110	1,540.0	868.0	0.048	672.0	43.6
				Feb. 15	1,080	0.114	1,231.2	702.0	0.049	529.2	43.0
26	8	52	50.5	Mar. 6‡	435	0.077	335.0	130.5	0.047	204.5	61.0
				" 8‡	495	0.075	371.3	104.0	0.054	267.3	72.0

* Mother diabetic.
† This urine gave a slight qualitative sugar test.
‡ These urines showed traces of albumin.

In the work of Benedict, Osterberg, and Neuwirth (1), it was stated that a total sugar output of 1.5 gm. per day should be regarded as the maximum for the normal adult on an ordinary diet and that if this figure should be revised, the revision would probably be downward. This prediction was well founded, in view of the figures reported in this paper—a maximum total sugar output of 1.38 gm., with an average figure of 0.94 gm. for 24 hours.

Of the subjects reported in Table II, one shows normal figures. Subject 24, however, shows on 2 of the 4 days recorded, figures

for the total sugar considerably higher than those in Table I; the fermentable sugar, however, is greater on all 4 days than is found in Table I. Whether this is due to a "diabetic tendency" or to the extremely liberal diet, is an open question.

Subject 25, whose urine frequently gives a slight qualitative sugar test, shows figures for total sugar which are somewhat higher than those of Table I and the non-fermentable sugar on both days was 43 per cent of the total sugar, a figure lower than obtains for the normals. It should also be noted that the fermentable sugar of this subject (702 to 868 mg.) is well above the maximum figure for this substance found for the normal (488 mg.).

Shaffer and Hartmann (6) have reported that results by their technique show that normal urines contain practically no fermentable sugar. Sumner (7) disagrees with this statement; there is also some work on infants (8, 9) which does not coincide with Shaffer and Hartmann's findings. The latter publish only percentage figures for total sugar; these figures are very low, compared with those given in the present paper. In all cases reported in this paper the results obtained after fermentation are lower than those found before fermentation, showing that normal urine does contain a reducing fermentable substance.

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THE NITROGEN DISTRIBUTION OF PROTEINS EXTRACTED BY 0.2 PER CENT SODIUM HYDROXIDE SOLUTION FROM COTTONSEED MEAL, THE SOY BEAN, AND THE COCONUT.

BY W. G. FRIEDEMANN.

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The determination of the nitrogen distribution of the proteins of cottonseed meal, the soy bean, and the coconut precipitable by slightly acidifying the alkaline extract was the object of this investigation.

The proteins were extracted by shaking the fat-free meal (60 mesh sieve) with a small amount of 0.2 per cent sodium hydroxide solution and several drops of alcohol for 5 minutes, centrifuging the extract, and shaking the residual meal for several hours with a small amount of dilute sodium hydroxide solution. The proteins were precipitated by acidifying the alkaline extract to 0.1 per cent acidity with dilute acetic acid. 4 gm. of the precipitated proteins were taken for the determination of the nitrogen distribution.

Soluble Nitrogen.—When alcohol was added to the concentrated unprecipitated nitrogen fraction no precipitate formed, with the exception of a small amount from the soy bean which was added to the precipitable proteins of the soy bean. When the soluble nitrogen fraction was dialyzed no precipitate formed. Sebelien[1] observed that the lactoglobulin of milk cannot be completely precipitated by dialysis or by dilute acetic acid.

Before hydrolysis with hydrochloric acid the amino nitrogen of the soluble nitrogen of cottonseed meal was 6.25 cc. at 21°C. and 741 mm., and after hydrolysis, 17.20 cc. at the same tempera-

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[1] Sebelien, J. *Z. physiol. Chem.*, 1885, ix, 448.

TABLE I.

Extraction of the Proteins with 0.2 Per Cent Sodium Hydroxide.

	Precipitated N.	Soluble N.	Residual N.
Cottonseed meal...................	62.0	19.8	18.2*
Soy bean........................	82.5	10.2	7.3†
Coconut.........................	78.3	10.9‡	10.8
Milk powder.....................	83.8	16.2	0.0

* Osborne and Vorhees (Osborne, T. B., and Vorhees, C. G., *J. Am. Chem. Soc.*, 1894, xvi, 785) state that 11.4 per cent of the nitrogen of cottonseed is insoluble both in salt and alkali.

† Muramatsu (Muramatsu, S., *J. Tokyo Chem. Soc.*, 1920, xli, 311; abstracted in *Chem. Abstr.*, 1920, xiv, 3265) states that 7.9 per cent of the nitrogen of the soy bean was not extracted with dilute alkali.

‡ Precipitated by phosphotungstic acid, 54 per cent of the soluble nitrogen or 5.9 per cent of total nitrogen.

TABLE II.

Nitrogen Distribution of the Protein of Cottonseed Meal (Van Slyke Method).†*

	0.2 per cent NaOH average precipitate.	5 per cent Ba(OH)₂ average precipitate.	Average globulin.
Amide N................................	10.54	10.47	10.38
Humin N absorbed by lime..............	1.88	2.00	1.24
Humin N in amyl alcohol extract........	0.21	0.18	0.11
Cystine N..............................	1.11	1.25	0.95
Arginine N.............................	23.48	24.04	22.74
Histidine N............................	4.94	5.49	6.24
Lysine N...............................	5.10	4.52	4.55
Amino N of filtrate.....................	51.26	51.03	51.87
Non-amino N of filtrate.................	2.15	1.42	2.07
Total N, *gm*...........................	0.38	0.40	0.58
N in protein (ash- and H₂O-free), *per cent*	15.98	16.75	18.2‡
Pentosans, *per cent*......................	3.3		

* Corrected for solubilities of bases.

† Van Slyke, D.D., *J. Biol. Chem.*, 1911–12, x, 15; 1915, xxii, 281.

‡ Osborne and Vorhees (Osborne, T. B., and Vorhees, C. G., *J. Am. Chem. Soc.*, 1894, xvi, 785) obtained 18.64 per cent nitrogen in the globulin they prepared. Also Osborne, T. B., The vegetable proteins, Monographs on Biochemistry, London, 1909.

ture and pressure in the same volume of solution. A solution containing 0.26 gm. of soluble nitrogen obtained from the coconut gave 4.9 cc. of amino nitrogen before hydrolysis and 50 cc. of amino nitrogen after hydrolysis at the same temperature and pressure. These results show that proteins are present in the soluble nitrogen fraction.

TABLE III.

Nitrogen Distribution of Soy Bean and Coconut Protein (Van Slyke Method).

	Soy bean.		Coconut.	
	0.2 per cent NaOH average precipitate.*	Average glycinin.†	0.2 per cent NaOH precipitate.*	
			I	II
Amide N.........................	11.31	12.19	7.40	7.52‡
Humin N........................	1.84	0.97	2.08	1.50
Cystine N.......................	1.04	0.80	0.86	0.70
Arginine N......................	14.57	15.35	28.60	28.67§
Histidine N.....................	5.92	2.38	4.88	5.60
Lysine N........................	8.26	10.27	4.56	4.99
Amino N of filtrate.............,	54.32	55.18	‖	47.18
Non-amino N of filtrate..........	2.71	2.93		2.94
Total N, *gm*.....................	0.487		0.5235	0.5056
N in protein, *per cent*.............	15.99	16.94	15.68	
Pentosans, *per cent*................	1.04		3.01	
N extracted, *per cent*..............			50 and 65	78.00

* Corrected for solubilities.

† Jones, D. B., and Waterman, H. C., *J. Biol. Chem.*, 1921, xlvi, 461.

‡ Osborne and Harris (Osborne, T. B., and Harris, I. F., *J. Am. Chem. Soc.*, 1903, xxv, 348) obtained 7.3 per cent amide nitrogen in coconut globulin.

§ Johns, Finks, and Gersdorff (Johns, C. O., Finks, A. J., and Gersdorff, C. E. F., *J. Biol. Chem.*, 1919, xxxvii, 149) obtained 15.92 per cent arginine nitrogen in coconut globulin.

‖ Total nitrogen of filtrate 51.35 per cent.

The 5 per cent barium hydroxide solution to which 1 per cent alcohol was added by volume extracted 74.3 per cent of the total nitrogen of cottonseed meal. The protein precipitated from the 0.2 per cent sodium hydroxide extract and the precipit-

able protein from the 5 per cent barium hydroxide extract had the same nitrogen distribution as that of the globulin.

The nitrogen distribution of the precipitable proteins in the 0.2 per cent sodium hydroxide extract of the soy bean shows a somewhat similar distribution to that of the globulin (glycinin).

No difference in the nitrogen distribution of precipitated protein containing 50, 65, and 78 per cent of the total nitrogen of the coconut was observed. The protein of the coconut precipitated from the 0.2 per cent sodium hydroxide extract by acidifying with dilute acetic acid differs markedly from coconut globulin in the arginine nitrogen value.

SEPARATE ANALYSES OF THE CORPUSCLES AND THE PLASMA.

By HSIEN WU.

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In spite of the varied functions of the different components of blood the value of separate analyses of the corpuscle and the plasma has been little appreciated. Unless distribution of a substance within the blood is known and the percentage of the corpuscles is taken into account, the data obtained from the whole blood analysis admit of no strictly scientific treatment. In the transport of material to and from the tissues both the corpuscles and the plasma may play a part; but their actual passage into or out of the circulation is effected only through the plasma. Hence for the solution of problems in connection with absorption, excretion, or similar processes, a knowledge of the concentration in the blood as a whole is insufficient, and the determination of the concentration in the plasma is a necessity.

Even in clinical practice where the whole blood analysis has proved a useful method of diagnosis and prognosis, separate analyses of the corpuscles and the plasma would afford a more reliable index of certain pathological conditions. For, if the distribution of a substance within the blood is unequal, the concentration in the blood as a whole will fluctuate with the percentage of the corpuscles, although the concentration in the plasma and and that in the corpuscles may remain unaltered.

The distribution of the better known water-soluble non-protein constituents has been a subject of considerable investigation. It is tacitly assumed by nearly all investigators that the results of the analysis of the corpuscles and the plasma obtained in the usual way truly represents the relation that exists in the circulating blood. Recently, Falta and Richter-Quittner (1) advanced

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the view that in the circulating blood the non-protein nitrogenous substances, the sugar, and the chloride occur only in the plasma, and that these substances enter the corpuscles only when they are injured by sodium oxalate, fluoride, or by other manipulations prior to the separation of the corpuscles from the plasma. In explaining the unusual findings upon which they based their conclusion, they referred to the use of hirudin as the *punctum saliens* of their research, believing that this anticoagulant, unlike the oxalate or fluoride, did not injure the corpuscles. The experimental data presented by these authors are, however, of such a character as to indicate faulty technique,[1] and their findings have failed to be confirmed by some Danish investigators (2, 3, 4). The view of Falta and Richter-Quittner cannot, therefore, be accepted at present.

The results of the study on distribution obtained by other investigators are of considerable interest. The total non-protein nitrogen (5, 6, 7), the amino-acids (5, 6, 8, 9, 10), and the creatine (6, 7, 11) are localized in the corpuscles, the urea (3, 5, 6, 12, 13, 14) and the creatinine (6, 7, 11) are equally distributed, while the uric acid (15, 16) is sometimes more and sometimes less concentrated in the plasma. The distribution of sugar (1, 17, 18) has long been a disputed question; in the case of human blood the preponderance of evidence now points to an approximately equal distribution. The chloride, the only inorganic constituent touched upon in the present paper, is more abundant in the plasma (19).

Unfortunately, most of the studies on distribution were conducted on the blood of different species of animals and the data at hand do not lend themselves to correlation. Moreover, parallel analyses were made not of the corpuscles and the plasma, but of the whole blood and the plasma, frequently neglecting to mention the percentage of the corpuscles. Where this percentage is given the concentration in the corpuscles can, of course, be computed. But it is obvious that with methods capable of only moderate degrees of accuracy slight differences between the concentration

[1] For instance, they reported in some cases as low as 9 mg. of non-protein nitrogen per 100 cc. of whole blood which is lower than the lowest figure for urea N we have ever found.

in the corpuscles and that in the plasma can be shown only by direct analyses of the corpuscles and the plasma.

Folin and Wu (20) developed some time ago a system of blood analysis in which the blood proteins are removed with tungstic acid, yielding a filtrate suitable for the determination of non-protein nitrogen, urea, uric acid, creatine, creatinine, and sugar, and, as shown recently by Whitehorn (21), also for the determination of chloride. It is, of course, very easy to extend that system so as to include separate analyses of the corpuscles and the plasma, and by this extension systematic studies on distribution will be facilitated, and the clinical application of the knowledge gained by such studies will be encouraged.

In applying the tungstic acid to the analyses of the corpuscles and the plasma we have required that the procedure employed must permit the quantitative recovery of at least 10 mg. each of uric acid and creatinine added to 100 cc. of plasma or corpuscles and that the non-protein nitrogen figures must be comparable with those of the whole blood. The latter requirement was deemed necessary in view of the fact that the non-protein nitrogen is not a very definite quantity but depends rather on the kind and amount of the precipitant used. It was found as a matter of fact, however, that the non-protein nitrogen values of the corpuscles and of the plasma obtained with different amounts of tungstic acid were substantially the same when the amount of this precipitant used was above a certain minimum. Table I gives the result of an experiment on this point. Table II shows that the non-protein nitrogen values for the whole blood, calculated from those of the corpuscles and the plasma, agree with those determined directly.

Instead of using tungstate and sulfuric acid solutions of different strengths for the corpuscles and for the plasma, it has seemed convenient to use the same 10 per cent tungstate solution and the $\frac{2}{3}$ N sulfuric acid and to retain the 1:10 dilution for the corpuscles and the plasma as for the whole blood. The method is as follows.

The oxalated blood is centrifuged in graduated tubes until the volume of the corpuscles remains constant. The length of time required for this has been determined previously. After noting the volume of the whole blood and that of the corpuscles,

TABLE I.

Experiment Showing Effect of Different Dilutions and Different Amounts of Tungstic Acid on Non-Protein Nitrogen of Corpuscles and Plasma.

Composition of precipitated mixture.	$\dfrac{\text{Volume of tungstate}}{\text{Volume of corpuscles or plasma}}$	Dilution.	Non-protein nitrogen per 100 cc.
			mg.
5 cc. corpuscles + 25 cc. H_2O + 10 cc. 10 per cent tungstate + 10 cc. $\frac{2}{3}$ N sulfuric acid..............................	2	1:10	50
4 cc. corpuscles + 26 cc. H_2O + 10 cc. 10 per cent tungstate + 10 cc. $\frac{2}{3}$ N sulfuric acid..............................	$2\frac{1}{2}$	2:25	49
6 cc. corpuscles + 24 cc. H_2O + 10 cc. 10 per cent tungstate + 10 cc. N sulfuric acid....................................	$1\frac{2}{3}$	3:25	51
5 cc. corpuscles + 75 cc. H_2O + 10 cc. 10 per cent tungstate + 10 cc. $\frac{2}{3}$ N sulfuric acid....................................	2	1:20	49
$7\frac{1}{2}$ cc. corpuscles + $22\frac{1}{2}$ cc. H_2O + 10 cc. 10 per cent tungstate + 10 cc. $\frac{2}{3}$ N sulfuric acid....................................	$1\frac{1}{3}$	3:20	Incomplete coagulation.
10 cc. plasma + 80 cc. H_2O + 5 cc. 10 per cent tungstate + 5 cc. $\frac{2}{3}$ N sulfuric acid..	$\frac{1}{2}$	1:10	35
8 cc. plasma + 82 cc. H_2O + 5 cc. 10 per cent tungstate + 5 cc. $\frac{2}{3}$ N sulfuric acid...	$\frac{5}{8}$	2:25	34
12 cc. plasma + 78 cc. H_2O + 5 cc. 10 per cent tungstate + 5 cc. $\frac{2}{3}$ N sulfuric acid..	$\frac{5}{12}$	3:25	34
10 cc. plasma + 30 cc. H_2O + 5 cc. 10 per cent tungstate + 5 cc. $\frac{2}{3}$ N sulfuric acid..	$\frac{1}{2}$	1:5	34
15 cc. plasma + 25 cc. H_2O + 5 cc. 10 per cent tungstate + 5 cc. $\frac{2}{3}$ N sulfuric acid...	$\frac{1}{3}$	3:10	Incomplete coagulation.

carefully pipette off the plasma without disturbing the corpuscle layer. This is best done by means of a pipette connected at the upper end with soft rubber tubing. Measure a convenient volume of the plasma, dilute with 8 volumes of water, and then add $\frac{1}{2}$ volume each of 10 per cent sodium tungstate solution and $\frac{2}{3}$ N sulfuric acid. Stopper the flask and shake.

Remove the plasma that remains above the corpuscle layer as completely as possible. Insert a blood pipette (20) into the corpuscle layer and take out a convenient volume. Lake it with 5 volumes of water and after thorough rinsing of the pipette with the corpuscle solution add 2 volumes each of the tungstate solution and the sulfuric acid. Stopper the flask and shake. The amount of plasma which cannot be removed from above the cor-

TABLE II.

Non-Protein Nitrogen of Corpuscles, Plasma, and Whole Blood.

Source.	Corpuscles.	Non-protein nitrogen per 100 cc.			
		Corpuscles.	Plasma.	Whole blood.	
				Determined.	Calculated.
	per cent	*mg.*	*mg.*	*mg.*	*mg.*
Sheep 1............	20	53	34	39	38
" 2............	26	52	36	40	40
Chicken 1.........	38	90	24	48	49
" 2.........	11	60	10	17	16

puscle layer is less than 0.1 cc., and if it is desired to economize the material the whole corpuscle layer may be used for analysis without appreciable error. It is then simply washed into an Erlenmeyer flask with 5 volumes of water followed by the required amounts of tungstate and sulfuric acid.

The precipitated corpuscles and plasma may be filtered immediately. The precipitated plasma should be poured on the filter, slowly at first to allow the wetting of the filter paper before any filtrate has passed through. If for any reason the precipitation is incomplete and the filtrate is turbid, the analysis can be saved by adding a few drops of normal sulfuric acid to the precipitate mixture. The plasma and corpuscle filtrates, like the filtrate of the whole blood, are perfectly clear, only faintly acid,

and suitable for the determination of all constituents included in the system of blood analysis.

The method described above has been applied to a number of cases of normal human blood. These were obtained from normal persons taking the Wassermann test and from patients suffering from slight external injury such as frost-bitten fingers and lacerated toes. The analyses included total non-protein nitrogen, urea, uric acid, amino-acids, creatine, creatinine, sugar, and chloride. The amino-acids were determined by a new method to be published shortly from the Biochemical Laboratory of the Harvard Medical School as a supplement to the system of blood analysis.

The results of the analyses, arranged in the order of increasing urea concentration of the plasma, are shown in Table III. They constitute in the main a confirmation of previous findings, and are intended primarily for illustration. They represent, however, the first series of comprehensive analyses of the corpuscles and the plasma of human blood and some discussion may therefore not be amiss.

Urea is about equally distributed, but the concentration in the plasma is sometimes a little higher than that in the corpuscles. The average value for the plasma is 19.3 mg. per 100 cc. as against 17.1 for the corpuscles. One may thus wonder whether in the determination of the so called Ambard coefficient or similar constants more consistent values would be obtained if the urea in the plasma instead of that in the whole blood were determined.

Contrary to the finding of Bornstein and Griesbach (16) we find that concentration of uric acid in the plasma is always higher than that in the corpuscles. The values of the ratio of the former to the latter are about 2 on the average. The finding of the Hamburg investigators that uric acid is sometimes higher in the corpuscles is probably an error, although we have not studied a large number of pathological cases[2] to exclude that possibility.

In view of the uncertainty in the determination of creatine and creatinine in the whole blood or rather in the corpuscles, as shown by Hunter and Campbell (11), any discussion on the distribution

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[2] We have studied the distribution of uric acid in a number of pathological cases. The results, not here reported, are similar to those in the case of normal blood.

TABLE III.

Non-Protein Nitrogenous Substances, Sugar, and Chloride in Corpuscles and Plasma of Normal Human Blood.

Per 100 cc.

No.	Corpuscles.	Total non-protein N.		Urea N.		Uric acid.		Total creatinine.		Preformed creatinine.		Amino-acid N.		Sugar.		NaCl.	
		Corpuscles.	Plasma.	Corpuscles.	Plasma.	Corpuscles.	Plasma.	Corpuscles.	Plasma.	Corpuscles.	Plasma.	Corpuscles.	Plasma.	Corpuscles.	Plasma.	Corpuscles.	Plasma.
	per cent	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	33	44	20	12	13	1.2	3.0	9.0	1.4	2.8	1.4	9.1	4.5	109	95	330	624
2	53	39	23	12	14	1.7	3.4	6.8	1.4	2.5	1.3	11.4	5.8	101	115	284	577
3	53	53	23	13	15	1.8	4.0	7.1	1.4	2.6	1.3	10.3	5.4	93	83	337	676
4	50	45	25	13	15	1.5	3.4	9.0	1.3	2.9	1.4	9.1	1.6	100	105	300	575
5	52	49	30	18	18	1.7	3.6	9.2	1.5	2.8	1.3	7.7	5.5	80	76	241	560
6	39	52	26	18	18	3.2	4.8	7.2	1.4	3.0	1.5	9.6	5.0	89	95	290	580
7	48	51	29	18	19	2.5	4.0	6.2	1.4	2.7	1.4	10.8	6.4	100	105	332	630
8	48	54	33	18	19	1.4	2.3	8.8	1.5	2.2	1.0	9.2	7.5	91	89	332	602
9	48	47	26	15	19	2.2	3.6	8.4	1.3	2.1	1.0	8.9	4.5	121	133	270	590
10	52	45	26	18	19	1.5	4.5	9.0	1.7	2.2	1.1	10.0	5.0	91	91	340	670
11	54	47	30	17	20	1.6	2.7	9.7	1.5	2.6	1.4	9.8	6.0	109	115	337	660
12	53	48	29	18	20	1.7	3.7	9.6	1.5	2.6	1.4	·9.2	5.5	103	107	312	630
13	57	46	35	16	21	1.7	3.8	9.3	1.4	2.8	1.3	9.4	6.2	100	100	313	629
14	50	45	29	18	21	2.0	4.5	7.9	1.5	1.9	1.0	8.9	6.0	87	95	292	583
15	47	48	29	16	21	3.8	5.7	10.2	2.1	1.8	1.0	10.5	5.4	106	100	302	600
16	40	56	31	22	22	2.5	5.0	9.0	1.4	3.0	1.4	10.0	6.6	93	100	345	675
17	38	61	27	21	22	2.0	4.8	8.5	1.4	2.8	1.4	9.0	4.0	98	112	331	652
18	47	55	35	21	23	1.4	3.3	9.5	1.3	2.8	1.4	8.6	6.1	100	110	326	616
19	48	54	36	18	23	1.8	3.9	6.8	1.5	1.6	0.8	8.2	5.0	110	114	313	604
20	45	48	34	20	23	1.3	4.5	7.1	1.5	2.0	0.9	9.7	5.3	115	123	270	570
rage....	47.8	49.3	28.8	17.1	19.3	1.93	3.92	8.32	1.47	2.48	1.24	9.47	5.52	99.9	103.2	309.7	615.2

of these constituents would seem unwarranted. It is clear, however, from Table III that the plasma is practically free from creatine. The average creatine content of the corpuscles is 5.84 mg. (as creatinine) per 100 cc., whereas that of the plasma is only 0.23 mg. This striking difference brought out by direct analysis of the corpuscles confirms and supplements the conclusion reached by Hunter and Campbell and by Wilson and Plass (7) that the creatine is chiefly contained in the corpuscles.

It may be of interest to point out also that the apparent creatinine content of the corpuscles is just twice that of the plasma.

The amino-acid content of the corpuscles averages almost twice that of the plasma. Similar distribution has been found in dogs (9), but so far as we are aware no figure has been reported for human blood.

The concentration of sugar in the plasma is usually a little higher than in the corpuscles, but at times the reverse may be true. However, the difference in concentration in such cases is often so small as to be within the limits of error.

The concentration of chloride in the plasma is about twice that in the corpuscles.

There is another interesting point which the present study has brought out. It is probably a recognized fact that the non-protein nitrogen in the whole blood is not wholly accounted for by the known constituents. A normal human blood contains on the average:[3]

	Per 100 cc. mg.
Urea N	17.10
Uric acid N	0.78
Creatinine N	0.47
Creatine N	1.30
Amino-acid N	7.13
Total non-protein N calculated	26.78
" " N determined	35.60
Undetermined N	8.80

[3] These figures, with the exception of the amino-acid N, are taken from a paper by Hammett (Hammett, F. S., *J. Biol. Chem.*, 1920, xli, 599). The amino-acid N figure is taken from Bock (10). That given by Hammett is 4.9 which seems too low.

There are about 9 mg. of nitrogen not accounted for in the whole blood. This question has been discussed by some authors, but so far as we are aware no satisfactory explanation has yet been offered.

Can the undetermined nitrogen be due to the incomplete removal of proteins? It is well known that the non-protein nitrogen values obtained with different methods do vary appreciably, and there are doubtless some protein precipitants which leave traces of protein in the filtrate. But the tungstic acid filtrate gives as low non-protein nitrogen as can be obtained with any other method without losing the known constituents and it is improbable that as much as 9 mg. of protein nitrogen can escape precipitation.

TABLE IV.

Distribution of Non-Protein Nitrogenous Constituents between Corpuscles and Plasma.

Constituent.	Corpuscles.	Plasma.
Urea N..	17.10	19.30
Uric acid N...................................	0.64	1.31
Total creatinine N............................	3.10	0.55
Amino-acid N.................................	9.47	5.52
Total non-protein N calculated	30.31	26.68
" " N determined	49.3	28.8
Undetermined N.............................	19.0	2.1

The present study on the distribution seems to throw much light on this question. It will be noted in Table IV, which represents the averages of Table III, that the non-protein nitrogen in the plasma is within limits of error[4] all accounted for and that the undetermined nitrogen is all contained in the corpuscles. This localization shows that the undetermined nitrogen is not due to incomplete precipitation of the proteins, for otherwise we should expect the plasma also to contain some of it.

To what substance then can be ascribed this undetermined nitrogen which amounts to almost 20 mg. per 100 cc. of corpuscles?

[4] There are, of course, traces of non-protein nitrogenous substances other than those determined.

In the corpuscles there are, on the one hand, the amino-acids, and on the other hand, the proteins. In the living protoplasm of the corpuscles, as in all living cells, there is a metabolic equilibrium between the amino-acids and the proteins with all the intermediates; namely, the peptides and peptones. The presence of peptides and peptones in the corpuscles is indeed very probable, and pending further experimental evidence the undetermined nitrogen in the corpuscles may be regarded as such.

Deductions Concerning Clinical Blood Analysis.—Since the undetermined nitrogen is all contained in the corpuscles and the amount of amino-acids in the corpuscles is larger than that in the plasma, the non-protein nitrogen of the whole blood will fluctuate with the content of the corpuscles in these constituents. As the latter is not known to vary with renal efficiency, the non-protein nitrogen of the whole blood would not be a reliable index of retention. The determination of the non-protein nitrogen of the plasma should therefore be preferred to that of the whole blood. The approximately equal distribution of sugar and urea justifies the usual practice of their determination in the whole blood, although their determination in the plasma is theoretically better.

The distribution of uric acid is so different and variable that one may wonder whether the content in the whole blood, the corpuscles, or the plasma is the most valuable for practical purposes. In the absence of other information the concentration in the plasma would furnish the most useful data.

Hunter and Campbell (11) have shown that the only substance in the plasma capable of simulating the reaction for creatinine is glucose and its influence upon the determination of creatinine is too small to have much practical importance, whereas the corpuscles contain other interfering substances which cause high results. Creatinine should therefore be determined in the plasma rather than in the whole blood.

There is thus abundant reason for substituting plasma analysis for whole blood analysis.

SUMMARY.

A method is described for the preparation of protein-free filtrates of the corpuscles and the plasma suitable for all determinations included in the system of blood analysis of Folin and Wu.

Sample analyses of the corpuscles and the plasma of normal human blood are given and the points of interest brought out by these analyses are discussed.

It is recommended that plasma analysis be substituted for whole blood analysis.

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A NEW COLORIMETRIC METHOD FOR THE DETERMINATION OF PLASMA PROTEINS.

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In connection with the study of a certaih blood reaction in cases of kala-azar (1), we had occasion to determine the different plasma proteins. The refractometric method of Robertson (2) or the recent method of Cullen and Van Slyke (3) based on nitrogen determinations would have served our purpose; but the lack of certain facilities required in these methods compelled us to devise some simpler technique adapted to the equipment at our disposal. As a result of our endeavor we have developed a new colorimetric method which is much simpler than and, we believe, fully as accurate as, any of the other methods.

There are two steps in the determination of the plasma proteins; *viz.*, (*a*) their separation from each other, and (*b*) their quantitative estimation. In the first step we have followed, in the main, the procedure of Cullen and Van Slyke. The isolation of the fibrin by recalcification of the oxalated plasma under the conditions prescribed by these authors is very satisfactory, and we have further simplified the process by whipping the fibrin out of the jelly, thus obviating the necessity of washing. For the precipitation of the globulin they used ammonium sulfate at half saturation. In our study we have found that saturation with magnesium sulfate gives the same results as half saturation with ammonium sulfate, and either process can be used in the new colorimetric method. In the application of this method to clinical cases, however, we have used the ammonium sulfate process which is somewhat more convenient. The magnesium sulfate process was used only when parallel determinations by the Kjeldahl method were desired.

In the second step we make use of the color reaction (4) of proteins with phospho-18-molybdictungstic acid (phenol reagent). Proteins in solution react with this reagent, due largely to the tyrosine which they contain. We have made no experiment to determine what the color produced by the proteins quantitatively represents. But since this chromogenic value is a constant for any given protein, the intensity of the color produced under definite conditions can be used as a measure of the amount of the same protein.

Solutions of pure serum globulin or albumin can, of course, be used for the standard. But as these are laborious to prepare and difficult to keep, we have used exclusively a solution of tyrosine in our work. A convenient standard is made by dissolving 50 mg. of tyrosine in 250 cc. of 0.1 N HCl. We have observed no change in this solution in the course of 6 months and it may keep much longer. Under the conditions described below we have found for human plasma that 1 mg. of tyrosine[1] = 16.4 mg. of fibrin (N × 6.25), 25.2 mg. of globulin (N × 6.25), or 27.5 mg. of albumin (N × 6.25).

The tyrosine equivalents of the plasma proteins of different species of animals are probably different and should be determined when required. To do this it is only necessary to make parallel determinations on the same sample of plasma by the new colorimetric method and by the Kjeldahl method.

In the method which follows the fibrin and the albumin are determined directly, while the globulin is determined by the difference between the total serum proteins and the albumin. All the determinations can be made simultaneously and finished in 1 hour.

Determination of the Fibrin.—To 1 cc. of the plasma (from blood containing 0.2 to 0.6 per cent potassium oxalate) add 28 cc. of 0.8 per cent NaCl solution and 1 cc. of 2.5 per cent CaCl₂ solution. Mix and allow to stand undisturbed for 20 minutes. Break up the jelly by shaking slightly and transfer it to a dry filter. While filtering insert into the jelly a slender glass rod with a pointed end

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[1] We used Pfanstiehl tyrosine prepared by the Special Chemicals Company, Highland Park, Ill.

and whirl gently. All the fibrin will stick to the rod.[2] Slip the fibrin off the rod, and press it between dry filter paper to remove as completely as possible the adhering liquid. Transfer it to a 15 cc. centrifuge tube, add 4 cc. of 1 per cent sodium hydroxide. Place the tube in a boiling water bath and stir with a slender glass rod until the fibrin lump has completely disintegrated. The fibrin has now dissolved, leaving the calcium oxalate in suspension. Add 10 cc. of water, mix, and centrifuge. Transfer the supernatant liquid to a 25 cc. volumetic flask or graduated tube. Cool under the tap. Add 1 cc. of 5 per cent H_2SO_4, 0.5 cc. of phenol reagent, and dilute to about 20 cc. Add 3 cc. of 20 per cent Na_2CO_3 solution. Shake. Add 1 drop of ether to dissipate the foam, make up to volume, and mix. The standard is prepared as follows: Measure 1 cc. of the standard tyrosine solution into a 25 cc. volumetric flask or graduated tube, add 0.5 cc. of phenol reagent, dilute to about 20 cc., and finally add 3 cc. of 20 per cent Na_2CO_3 solution. Make up to volume and mix. The standard should, of course, be prepared at the same time as the unknown. Let stand for 15 minutes before making the color comparison.

Calculation.—If the standard is set at 20 and the reading of the unknown is R, then the amount of the apparent tyrosine determined is $\frac{20}{R} \times 0.2$ mg. Since 1 mg. of tyrosine = 16.4 mg. of fibrin, the amount of fibrin in 1 cc. of plasma is $\frac{20}{R} \times 0.2 \times 16.4$ mg. or the percentage of fibrin = $\frac{20}{R} \times 0.328$.

Determination of Albumin.—To 1 cc. of plasma add 9 cc. of $\frac{5}{8}$ saturated ammonium sulfate solution[3] or 9 cc. of saturated magnesium sulfate solution and 0.3 gm. of anhydrous magnesium sulfate. Mix and allow to stand for 30 minutes. Filter. Measure 1 cc. of the filtrate into a 15 cc. centrifuge tube. Add about

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[2] If any fibrin fails to stick to the rod it should be picked up with the tip of the rod. It requires no great skill to do this successfully. If the amount of the fibrin in the plasma is very high, say 0.8 per cent or more, the fibrin jelly will not shrink readily. In such a case it is necessary to use less of the plasma for the determination.

[3] This is made by diluting 555 cc. of saturated ammonium sulfate to 1 liter.

12 cc. of H_2O, 1 cc. of 10 per cent sodium tungstate solution, and then 1 cc. of $\frac{2}{3}$ N sulfuric acid. The amount of the tungstate solution and the sulfuric acid need not be exactly measured, but care must be taken to see that the volume of the acid used is at least equal to that of the tungstate solution. Stir thoroughly with a slender glass rod and centrifuge. Carefully decant off the supernatant liquid as completely as possible. (The volume of the wet precipitate usually amounts to about 0.5 cc. If it is much smaller than 0.5 cc., indicating low albumin, measure another cc. of albumin filtrate into the same tube, dilute with water and proceed as before.) Add to the precipitate in the centrifuge tube 1 cc. of sodium tungstate solution. Stir until the precipitate has dissolved, dilute with 13 cc. of H_2O, and add 1 cc. of H_2SO_4. Stir again, centrifuge, and decant off the supernatant liquid. This second precipitation is intended to remove the calcium and ammonium or magnesium so nearly completely that they cannot possibly interfere with the subsequent color reaction, although experience has shown that a single precipitation usually suffices. Add to the precipitate in the tube 10 cc. of H_2O and 1 or 2 drops (but no more) of 20 per cent Na_2CO_3. Stir until the precipitate has dissolved. Transfer the resulting solution to a 25 cc. volumetric flask or graduated tube. Rinse the centrifuge tube twice with 3 cc. of H_2O.. Add 0.5 cc. of phenol reagent and 3 cc. of 20 per cent Na_2CO_3 solution. Shake. Add 1 or 2 drops of ether to dissipate the foam. Make up to volume and mix. Prepare a standard as in the fibrin determination and read the color after 15 minutes.

Determination of Albumin and Globulin.—Measure 2 cc. of the filtrate in the fibrin determination into a 15 cc. centrifuge tube and proceed exactly as in the determination of albumin.

Calculation.—In the calculation it is to be noted that the solution used for the albumin determination is plasma diluted 1 to 10, while that used for the determination of albumin and globulin is plasma diluted 1 to 30. If 1 cc. of the albumin solution is used for the former determination and 2 cc. of the serum solution are used for the latter determination and the colorimeter readings are R_a and R_t respectively, the standard being set at 20, then the total apparent tyrosine in 1 cc. of serum $= 15 \times \dfrac{20}{R_t} \times 0.2$ mg.,

the apparent tyrosine of albumin in 1 cc. of serum $= 10 \times \frac{20}{R_a} \times 0.2$ mg., and the apparent tyrosine of globulin in 1 cc. of serum is

$$(15 \times \frac{20}{R_t} \times 0.2) - (10 \times \frac{20}{R_a} \times 0.2) = \frac{60}{R_t} - \frac{40}{R_a} \text{ mg.}$$

Since 1 mg. of tyrosine $= 25.2$ mg. of globulin $= 27.5$ mg. of albumin,

$$\therefore \text{ per cent of globulin} = \frac{\left(\frac{60}{R_t} - \frac{40}{R_a}\right) \times 25.2}{1,000} \times 100$$

$$= \left(\frac{6}{R_t} - \frac{4}{R_a}\right) \times 25.2$$

$$\text{per cent of albumin} = \frac{\left(10 \times \frac{20}{R_a} \times 0.2\right) \times 27.5}{1,000} \times 100 = \frac{20}{R_a} \times 5.44$$

EXPERIMENTAL.

Determination of the Tyrosine Equivalent of the Plasma Proteins.

Fibrin.—The fibrin in about 5 cc. of human plasma was isolated as described. It was placed in a 25 cc. volumetric flask and 20 cc. of 1 per cent NaOH were added. The flask was placed in boiling water with frequent shaking. When the fibrin lump had completely disintegrated the flask was cooled under the tap and the solution was made up to volume with 5 per cent H_2SO_4 and mixed. It was filtered to remove the calcium oxalate. 5 cc. of the filtrate were taken for the tyrosine determination as described above. The nitrogen was determined in 2 or 3 cc. of the filtrate by the micro Kjeldahl method (5). It was found in two experiments representing two samples of plasma that 1 mg. of tyrosine $= 16.8$ and 16.0 mg., respectively, averaging 16.4 mg. of fibrin (N $\times$ 6.25).

Albumin.—The globulin together with fibrin was precipitated from the plasma using magnesium sulfate as described. 1 cc. of the filtrate was taken for the tyrosine determination. 5 cc. of the filtrate were measured into a 10 cc. volumetric flask, diluted to volume, and mixed. 1 cc. of this diluted solution was measured into a Pyrex test-tube, 2 cc. of digesting mixture were added, and

the digestion was carried out in the usual way (5).   25 cc. of H_2O were added and the solution was cooled and transferred to a very large test-tube or small flask.   25 cc. of 10 per cent NaOH were then added and the ammonia was aspirated and determined by Nesslerization in the usual manner.   The ammonia in the reagents and the non-protein nitrogen of the plasma were deter-

TABLE I.

*Plasma Proteins in Normal and Pathological Blood.**

No.	Diagnosis.	Fibrin.	Total serum protein.	Albumin.	Globulin.
		per cent	per cent	per cent	per cent
1	Normal........................		6.50	4.55	1.95
2	"		6.55	4.80	1.75
3	"		7.51	5.28	2.23
4	"	0.222	6.62	4.75	1.87
5	"		7.54	4.89	2.65
6	Syphilis........................		8.04	4.63	3.41
7	"		9.37	4.95	4.42
8	Kala-azar........................		9.59	3.83	5.76
9	"		10.54	3.48	7.06
10	"		6.78	2.90	3.88
11	"		7.37	2.94	4.43
12	"	0.271	6.99	1.82	5.17
13	"	0.306	7.82	2.35	5.47
14	" treated..............	0.290	7.40	4.05	3.35
15	"	0.292	7.79	2.62	5.17
16	"		7.98	1.94	6.04
17	Streptococcus septicemia........		5.56	2.76	2.80
18	Amebic dysentery..............		4.24	2.90	1.34
19	Relapsing fever.................	0.485	5.57	2.58	2.99
20	Nephritis with edema...........	0.630	3.07	0.53	2.54
21	Anemia........................		7.50	5.00	2.50

* Some of the figures in this table have been reported elsewhere (1).

mined and corrections were made accordingly.   In five experiments representing five specimens of plasma it was found that 1 mg. of tyrosine = 27.6, 27.4, 27.5, 27.3, and 27.8 mg., respectively; averaging 27.5 mg. of albumin (N × 6.25).

Globulin.—1 cc. of the serum filtrate of the same plasma used for the albumin determination was measured into a Pyrex test-tube and the nitrogen was determined exactly as in the preceding ex-

periment. In another 2 cc. of the filtrate the tyrosine was determined. It was found by the method of calculation indicated above that 1 mg. of tyrosine = 24.9, 25.8, 24.7, 24.9, and 25.6 mg., respectively; averaging 25.2 mg. of globulin (N × 6.25).

It would appear from the values of tyrosine equivalents given above that the fibrin contains much more tyrosine than the albumin or the globulin. But this is not the case. We have observed that the chromogenic value of the albumin or the globulin is greatly increased by treating with NaOH for a few minutes or with Na_2CO_3 on long standing. The apparently high tyrosine content of the fibrin is no doubt due to the action of NaOH used to bring it into solution.

In Table I are shown some of the results obtained with the new method. It is not the purpose of the present paper to discuss the significance of the findings in those pathological cases reported here, but the deviation from normal is sometimes so marked that the clinical value of the determination of plasma proteins is apparent.

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STUDIES ON EXPERIMENTAL RICKETS.

XVI. A DELICATE BIOLOGICAL TEST FOR CALCIUM-DEPOSITING SUBSTANCES.

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PLATES 1 AND 2.

In our second communication (1) we described two diets (Lots 2638 and 2677) which we had used to prepare animals for a biological test for the calcium-depositing power of various food substances. This test depends on the power of a given substance to cause the reappearance of a provisional zone of calcification in epiphyseal cartilages of animals with very severe rickets, whose cartilages had been rendered calcium-free by faulty diets. We called this procedure the "line test," because in gross specimens or in histological preparations the new zone of calcification appears as a line of calcified tissue crossing an area which is free from lime salt deposits. Schmorl (2) showed that the first sign of healing in rachitic children was the reformation of the provisional zone of calcification on the epiphyseal side of the metaphysis. The value of the reaction is dependent upon the certainty with which a given diet will operate to produce bones in which the epiphyseal cartilage and the metaphysis are calcium-free. In other words, it is not sufficient that the diet shall produce rickets. It must cause an exaggerated form of florid rickets to develop without fail. Our results with the above diets were not entirely satisfactory, since now and again an animal would show some evidence of calcification of the epiphyseal cartilage. Furthermore, we

found that young rats on these diets did not grow enough to produce as severe rickets as we desired.   Many animals died in a short time because of the pronounced deficiencies of the diet (Lot 2677).   Moreover, rats on these diets showed a marked tendency to destroy and eat their companions in the cage.   This, of course, vitiated the experiments.

The data presented in the earlier papers of this series leave no room for doubt that certain faulty relations between three dietary factors, *viz.* calcium, phosphate, and an unidentified organic substance, lead to the development of the picture of rickets in young rats as it occurs in children.   When a diet contains a somewhat excessive amount of calcium, decidedly less than the optimal amount of phosphate, and very little of an organic substance which is especially abundant in cod liver oil, rats which are fed on it develop rickets (3).   We have pointed out that pronounced rickets develops only when the conditions are such as to permit young rats to grow.   When the faults in the diet are such as to cause rickets, the severity of the disease is increased if the food is such as to insure to the animal a certain amount of growth and to keep up its vitality.

A diet which proves highly satisfactory for the purpose in hand, in that young rats fed upon it have marked rickets with an epiphyseal cartilage and metaphysis uniformily free from calcium, is made up as follows:

Diet 3143.

Whole wheat kernel..33.0	100 gm. of this diet contains 1.221 gm.
" maize " ..33.0	of calcium, and 0.3019 gm. of phos-
Gelatin...............15.0	phorus.
Wheat gluten.........15.0	Weight ratio Ca:P = 4.04:1
NaCl................. 1.0	
CaCO₃............... 3.0	Atomic ratio Ca:P = 1:0.319

The proteins of this diet are of good quality and are abundant (about 33 per cent of the food mixture).   The amount of phosphorus in the mixture is distinctly below the need of the growing rat for this element.   100 gm. of this food contain 0.3019 gm. of phosphorus.   Our observations indicate that the optimal amount of phosphorus in the diet of the growing rat is not less than 0.4146 per cent.   It may be considerably higher.   The calcium

content of the food mixture is 1.221 gm.—approximately double that required for optimal growth and function. The content of fat-soluble A is very low but suffices to prevent the development of xerophthalmia, and to make growth possible for a time, other factors permitting. The content of the second organic factor supplied by certain fats (if this proves to be a distinct entity) is likewise very low.

Young rats which have grown rapidly to about 55 to 60 gm. (35 to 40 days old) are confined to Diet 3143 for a period of about 35 to 40 days (preparatory period). If the animals are allowed to remain too long on this diet they deteriorate physically and their hind legs become paralyzed. After being confined to the rickets-producing diet for from 35 to 40 days the rats have some difficulty in using their hind legs. The changes in the animals' gait are not so striking that they would be noticed except by one who is familiar with the habits and movements of the rat. The gait is tottering, and the hind quarters waver slightly from side to side. When the animal starts to move off rapidly it hops, usually favoring one hind leg. We have never failed to find the cartilages of the bones of animals in this condition calcium-free. The metaphysis is usually quite free from calcium salts but occasionally a few calcified areas are found.

When a group of rats are ready to be used for the test, some of them are given the substance which we desire to study, in their food, and the rest serve as control animals and continue to receive Diet 3143 unchanged. (The time during which the rats receive the substance which is being studied may be called the "test period.") The test animals and controls are killed and autopsied at the end of a designated time. It has been our custom to examine the distal end of the left femur and the proximal end of the left tibia for reformation of the provisional zone of calcification. Of the two bones the tibia is perhaps the better, since the epiphyseal line is straighter in this bone than in the femur, and the metaphysis wider.

The bones are split in two with a sharp scalpel and one-half of each is fixed in 10 per cent formaldehyde, decalcified in Müller's fluid, and embedded in parloidion. The other half of each bone is immersed in a 1 per cent solution of silver nitrate and exposed to sunlight or to the light of a Mazda lamp, and studied through a

binocular microscope. The slices which have been treated with silver nitrate may be cut into frozen sections or may be preserved as permanent gross specimens. For the latter purpose they are washed well with water, decolorized with a solution of sodium thiosulfite, rewashed and kept in formaldehyde.

The bones of these animals are very soft. The ends are very much enlarged. On section the cartilage of the epiphysis is irregular in depth and the epiphysis is separated from the diaphysis by a zone of white tissue (the metaphysis) which may be about 0.5 cm. deep. In the bones of the control animals or rats which give a negative "line test" there is no calcification of the epiphyseal cartilage, and little if any in the metaphysis (Fig. 1). The shaft is incompletely calcified. We have described these animals and their bones completely in another place (3). Rats which give a positive "line test" differ from the controls in having a broad, linear deposit of calcium salts on the metaphyseal side of the epiphyseal cartilage. The band, which may not be complete, is separated from the shaft of the bone by the depth of the metaphysis, and from the nucleus of ossification of the epiphysis by the depth of the epiphyseal cartilage. It can be seen on the freshly cut surface of an untreated bone as a yellow line which marks the epiphyseal border of the metaphysis. The deposit is blackened by silver nitrate in fresh or fixed gross specimens. It appears like a cross-section of a black honeycomb when it is examined with a binocular microscope. The metaphyses of these bones usually appear to be congested. This deposit is in the proliferative zone of the cartilage. It may extend completely across the bone or may be interrupted or fragmentary according to the activity of the calcium-depositing substance (Fig. 2). It is stained brown by silver nitrate and an intense blue by hematoxylin. It is separated from the calcified trabeculæ of the shaft by the width of the metaphysis. The honeycomb-like appearance of the line is explained by sections which show that the matrix of the cartilage only is calcified (Fig. 3). The cartilage cell is not. The deposit is granular.[1] Although the possibility of detecting this calcification in living

[1] In studying fresh, silvered preparations it must be borne in mind that a precipitate of silver is sometimes thrown down irregularly on the surface of the bone without relation to calcium deposits. If this precipitate is

children by the use of the x-ray has been recognized for sometime (4), Park and Howland (5) were the first to make use of it experimentally. They were able to show that cod liver oil would regularly induce calcification and healing in the bones of children with rickets. Since their experiments Huldschinsky (6) and others have demonstrated the curative power of ultra-violet light in the same way. When rats are used as subjects the picture on the x-ray plate may be confirmed by microscopic study of the bones.

The results obtained with this test are illustrated by the effect of the administration of cod liver oil. Within 5 days the addition of 2 per cent of cod liver oil to the food causes complete recalcification of the cartilage.   -

Cod liver oil added to the food in amounts equal to only 0.2 or 0.4 per cent of the food mixture does not cause the line of calcium phosphate to appear in 5 days. 0.6 per cent, on the other hand, causes the appearance of an irregular calcification in some of the bones in this time. We shall describe later the effects of small doses of cod liver oil repeated for shorter or longer periods in producing the reformation of the provisional calcified zone.

In conducting these tests it is necessary to keep accurate records of the food consumption by both test and control rats during the interval following the administration of the substance to be tested. This is so because starvation causes rats with rickets to deposit calcium salts in the epiphyseal cartilage, just as does cod liver oil (7). We have taken this precaution in all our tests. A positive test can be relied upon only when the animals eat enough.

Since the amount of and the relation between calcium and phosphorus in the food is a very important factor in determining the ability of the skeleton to grow normally, it is essential, when employing the test here described for the examination of a natural food (*e.g.* the leaves of plants, etc.), to modify the basal diet at

formed on the cartilage it may be mistaken for an irregular calcification. The regular honeycomb appearance of the true "line" of calcification distinguishes it. In case of doubt the bone may be treated with sodium thiosulfite. The blackness of the silvered calcium is unaffected by 2 to 3 minutes' treatment with the solution, but the precipitate will be dissolved at once. We have always controlled the results of the examination of fresh tissues by the study of microscopic sections. The results obtained by the use of the two methods have never failed to correspond.

TABLE I.

Rat No.	Age when put on diet.	Days on diet.	Weight at death.	Sex.	Control.	Days received diet plus cod liver oil.	Food eaten in 5 days.	Healing.
	days		gm.				gm.	
1178	60	70	73	♀		1.0 per cent cod liver oil for 5 days.	1*	+
1179	60	70	108	♀		0.6 " " " " 5 "	28	Irregular deposit.
1180	60	70	98	♀		0.4 " " " " 5 "	29	0
1181	60	70	117	♀		0.2 " " " " 5 "	29	0
1220	45	64	92	♂		1.0 " " " " 5 "	22	+
1348	45	49	107	♂		2.0 " " " " 5 "	36	+
1349	45	49	77	♂		2.0 " " " " 5 "	33	+
1350	45	49	90	♀		2.0 " " " " 5 "	29	+
1352	55	49	75	♀		2.0 " " " " 5 "	40	0
1323	40	39	73	♂	Control.		35	0
1334	50	57	105	♀	"		37	0
1335	50	57	85	♀	"		35	0
1336	50	57	130	♀	"		38	0
1224	55	70	86	♀	"		39	0
1225	35	70	100	♀	"		36	0
1354	45	54	110	♀	"		37	0
1337	50	47	135	♂	"		37	0
1355	45	54		♂	"		39	0

* Comparable to starvation.   Rat was paralyzed in hind legs and was in helpless condition.

the time of making the test, so that the added food will make no change in the inorganic composition of the diet as compared with that of the food in the preparatory period. In another paper in this series we show in some detail how important it is to adhere rigidly to the composition laid down for the diet used for the preparation of the animals for this test.

The test is applicable for the demonstration of the effect of light rays or other radiations, or other remedial agencies. It constitutes, therefore, a valuable aid to further experimental work in the field of bone pathology.

SUMMARY.

1. A diet is described which is low in phosphate and in an undetermined organic substance and relatively high in calcium. This diet uniformly causes the bones of animals fed on it to be free from calcium as regards the epiphyseal cartilages and the metaphyses. These bones show an exaggerated type of florid rickets.

2. The reappearance of the provisional zone of calcification after the addition of any substance to the rickets-producing diet (beginning healing of the rickets) constitutes a test for the calcium-depositing power of the substance (the so called "line test").

3. Cod liver oil to the extent of 2 per cent by weight of the diet causes the provisional zone of calcification to be reformed in these bones in 5 days (a positive "line test").

4. 0.4 per cent of cod liver oil fails to cause the provisional zone of calcification to reappear in the bones. 1 per cent of cod liver oil for 5 days does not cause so complete a calcium salt deposition as does 2 per cent.

5. Both test and control animals must be kept under the same conditions of illumination, and since starvation causes redeposition of calcium in the bones, records of the food consumption of the animals must be kept during the experiment.

6. The basal diet must be so modified when any natural foods are tested that the addition of the food to be examined will not change the inorganic content of the mixture used in making the test from that of the original diet used during the preparatory period.

7. This test may be used to determine the ability of any agency to heal rickets.
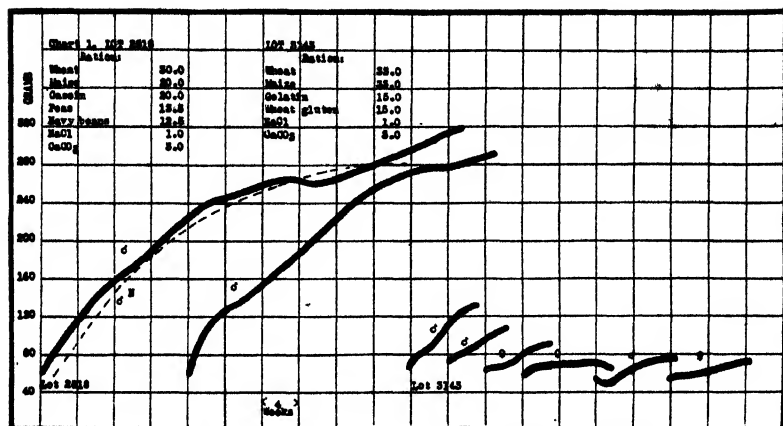
CHART 1. The curves of Lots 2818 and 3143 illustrate the importance of having an optimal ratio between calcium and phosphorus in the diet of the growing rat.

Lòt 3143 had a diet which contained an abundance of proteins (33 per cent) of good quality, but was too low in phosphorus and too high in calcium. The diet contained 1.221 gm. of calcium in 100 gm. This is about twice the optimal amount of this element. 100 gm. contained 0.302 gm. of phosphorus. This we know to be distinctly below the optimal amount, although the most favorable concentration of this element in the diet has not been accurately determined. In certain other diets we have been able to secure good nutrition when the food contained 0.415 per cent of phosphorus. We may safely assume that the diet under consideration is at least 30 per cent too low in this element. In addition to these defects the diet of Lots 3143 and 2818 was relatively deficient in fat-soluble A.

The diet of Lot 2818 differed essentially from that of Lot 3143 in its content of phosphorus. As respects protein, calcium, and fat-soluble A, they were essentially alike. Diet 2818 contained 0.4928 per cent of phosphorus, and 1.247 per cent of calcium. The calcium: phosphorus ratio in each of these rations were:

<table>
<tr><td align="center">*Lot 3143.*</td><td align="center">*Lot 2818.*</td></tr>
<tr><td>Atomic ratio ...Ca:P::1:0.319</td><td>Atomic ratio ...Ca:P::1:0.523</td></tr>
<tr><td>Weight ratio ....Ca:P::4.04:1</td><td>Weight ratio ....Ca:P::2.51:1</td></tr>
</table>

The diet of Lot 3143 contained only 61.25 per cent as much phosphorus as did the diet of Lot 2818. This difference in phosphorus content was sufficiently significant for the welfare of the growing rat to make the differences in growth seen in the contrasting curves of the two groups shown in the chart.

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EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. This figure shows the distal end of a section of the tibia of a control animal which had been fed our diet, No. 3143. Note the proliferative zone of cartilage (*C*) which is entirely free from calcium salts, and the wide calcium-free zone (*M*) between the cartilage and the end of the shaft (*D*) which contains partially calcified trabeculæ.

FIG. 2. This figure shows the distal end of the tibia of an animal which had given a positive line test. This animal had been fed diet, No. 3143, but had received 2 per cent of cod liver oil for 5 days before death. Note the "line" (*CA*) of newly deposited calcium in the proliferative cartilage. This line is a newly formed zone of provisional calcification.

PLATE 2.

FIG. 3. Higher magnification, × 300, of the newly formed provisional calcified zone (*CA*). This picture shows the calcification of the intercellular matrix of the proliferative cartilage. Some of the calcified cell capsules have been broken open and invaded by capillary vessels from the shaft.

Fig. 1.

Fig. 3.

(McCollum, Simmonds, Shipley, and Park: Studies on experimental rickets.　XVI.)
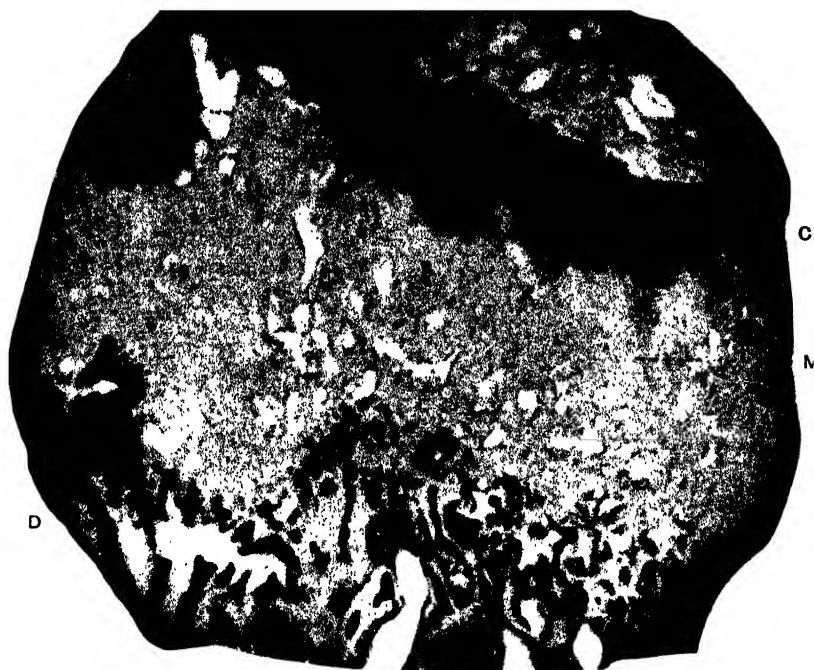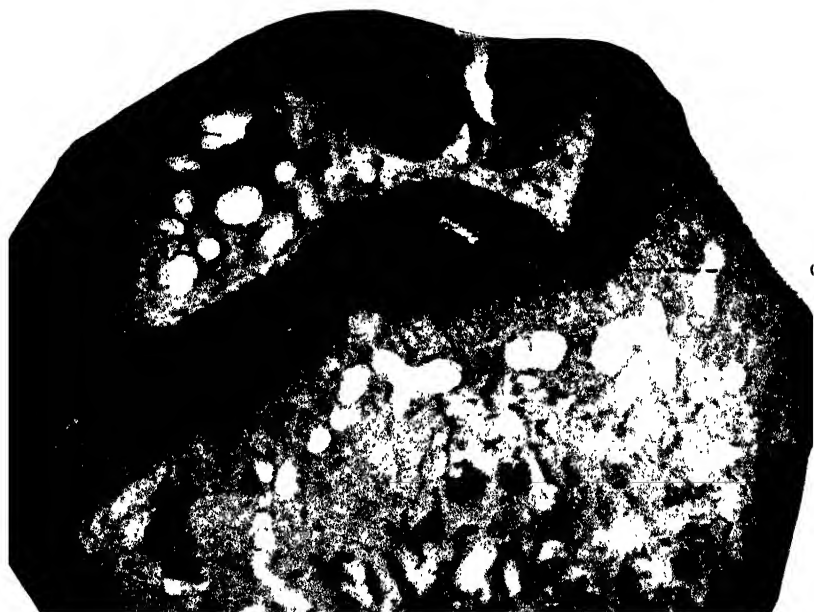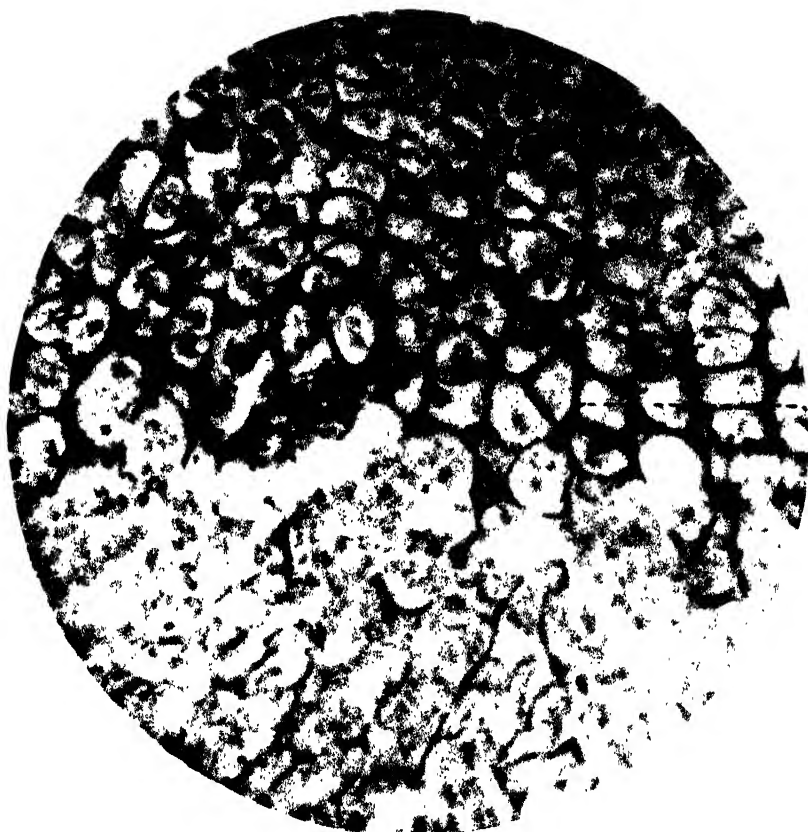
CALCIUM IN EGG-SHELL FORMATION.

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Domestication of the chicken has intensified its egg-laying power and it is not unusual for a White Leghorn hen to lay 300 eggs in 1 year. This is a large number when contrasted with the 22 to 26 eggs laid yearly by the jungle fowl, *Gallus ferrugineus*, the probable progenitor of the domestic fowl. For the production of eggs the Leghorn will require yearly approximately 750 gm. of CaO instead of the 32 gm. used by the jungle fowl for that purpose.

Common practice teaches that calcium in some concentrated form, such as oyster shell, must be added to the ordinary feeds fed to poultry in order to supply the large amount of calcium necessary for optimum egg production, and that grit is added to aid digestion.

In connection with an experiment dealing with the Ca, Mg, and P metabolism in the laying hen, six lots, each containing ten 7 month old White Leghorn pullets which came from the same parent stock, hatched the same day, and existing under identical conditions, were fed the following feeds:

Lot 1. Grains + tankage + no mineral material.
" 2. " + " + granite grit, *ad libitum*.
" 3. " + " + " " " " +oyster shell, *ad libitum*.
" 4. " + " + " " " " +limestone, *ad libitum*.
" 5. " + " + limestone, *ad libitum*.
" 6. " + " + rock phosphate, *ad libitum*.

Tankage (12.5 per cent of total fed) containing 6.4 per cent P_2O_5 was fed in the dry mash. The grit used contained 2.4 per cent CaO soluble in strong HCl.

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This experiment was started December 1, 1920, and ended August 1, 1921. During these 8 months the pullets were confined in houses and at no time allowed access to the ground, thereby excluding any chance of their obtaining calcium-containing materials from undesired sources. No milk or green foods were fed.

At the beginning of the experiment two representative birds were killed and after their femurs and tibias were dissected out and separately weighed, the CO_2-free ash and the CaO contained therein were determined. A hen of the same age, having received the same treatment as all other birds in this experiment up to December 1, 1920, but which had from that time until August 1, 1921, been allowed the normal freedom of a meadow and been fed the same as Lot 3, was killed and similarly dissected as were the early controls just mentioned. All the birds remaining in the six lots on that date were killed and treated in the same way.

A trap-nest record was kept of each bird confined. Among other things the CO_2-free ash and the percentage of CaO in this ash were obtained in a composite sample of the shells of the first three eggs laid each month by each hen.

The results in Table I, which represents averages, show that the total weights of the four large leg bones of the hens in Lots 1, 2, 3, 4, 5, and 6 were approximately the same, while that of the first control was lighter, being 8 months younger, and that of the second control was heavier, resulting from the different physical conditions governing the 8 months over which this experiment extended. The actual weight of the CO_2-free ash of the leg bones from Lots 1 and 2 was lighter than that from Lots 3, 4, 5, and 6 which received the concentrated calcium materials, and the ash from Lot 6 which received rock phosphate was heavier than that from any of the other lots. This agrees with results published by D'Anchald[1] in 1904 who obtained a heavier skeleton by adding daily 4 gm. of powdered bone per hen to an ordinary poultry feed.

It will be seen that Lot 1, which received no mineral matter in addition to the grains and tankage fed, laid 19.9 eggs per hen in 8 months which is slightly in excess of the number laid by

[1] D'Anchald, H., *J. agric. prat.*, 1904, vii, 619.

TABLE I.

Date.	Lot No.	Average total weight of four leg bones.	Average weight of CO₂-free ash in four leg bones.	Average per cent of CO₂-free ash in leg bones.	Average CaO in CO₂-free ash of leg bones.		Average number of eggs laid per lot per hen per in 8 months.	Average weight of CO₂-free shell ash in 1 egg per lot.	Weight of CaO in average shell of 1 egg per lot.	Per cent of CaO in average CO₂-free shell ash per lot.
		gm.	gm.	per cent	gm.	per cent		gm.	gm.	
1920										
Dec. 1	1st control.	22.34	5.63	25.2	3.09	54.9				
1921										
Aug. 1	1	30.36	7.97	26.4	4.33	54.3	19.9	1.84	1.79	97.47
" 1	2	31.95	8.80	27.6	4.79	54.4	38.8	1.98	1.93	97.47
" 1	3	31.30	10.59	33.8	5.79	54.7	52.2	2.45	2.39	97.68
" 1	4	30.73	10.18	32.5	5.61	55.2	52.6	2.42	2.36	97.53
" 1	5	30.54	10.69	34.7	5.88	55.0	59.6	2.54	2.48	97.59
" 1	6	31.12	11.20	36.0	6.17	55.0	18.1	1.81	1.77	97.69
" 1	2nd control.	36.98	11.94	32.3	6.57	54.9				

Lot 6 which received the rock phosphate *ad libitum*. This would seem to indicate, when considered with the previous statement, that the rock phosphate can be utilized by the hen in bone formation but not in the production of eggs, calcium being the limiting factor in the case under consideration. This is shown by the fact that the average weights of the CO_2-free ash and the CaO in the egg-shells of Lots 1, 2, and 6 are practically the same while those in Lots 3, 4, and 5 are larger. It is interesting to note that the percentages of CaO in the CO_2-free ash of the egg-shells is practically constant in all lots. Elsewhere[2] we have shown that the quantity of CaO in the contents of the eggs governed by these conditions is practically constant.

Lot 2 laid a few more eggs per hen than did Lot 1—probably due to the 2.4 per cent CaO available in the grit fed to Lot 2. The average number of eggs laid by each hen in Lots 3, 4, and 5 is approximately the same for each lot.

In briefly summing up these results it would seem that the hen can utilize the calcium in calcium carbonate for the production of both egg-shell and bones but that the calcium in tricalcium phosphate can only be utilized for the growth of bone and not for egg-shell production. Finally, calcium starvation is not the determining factor in the production of shell-less eggs.

[2] Buckner, G. D., and Martin, J. H., *J. Biol. Chem.*, 1920, xli, 195.

A METHOD FOR THE ESTIMATION OF TOTAL BASE IN URINE.

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The investigation of diurnal variations in the excretion of bases (with the exception of ammonia) has received practically no attention. While this might formerly have been due to the fact that suitable methods for the estimation of the important individual non-volatile bases (sodium, potassium, calcium, and magnesium) were not available, that is no longer so. The main deterrent appears to be the large amount of work required for the separate determinations of these four constituents in the many samples of urine involved in most short period metabolism experiments. When, as may often be the case, the information sought is the total equivalent of all four bases, rather than the amount of each one, the magnitude of the task is out of all proportion to the value of the final result, which includes the accumulated errors of the four individual analyses. Since, in the interpretation of the results of such investigations, the equivalent of the chloride content of the urine must ordinarily be subtracted, and since the remainder may be comparatively small, the micro methods so far described are not sufficiently precise to furnish figures for the sum of the bases that have the requisite degree of accuracy.

The method described here is designed to accomplish this. Up to the final step in the analysis, it is merely an adaptation of long used schemes for the separation of these bases from other substances. The urine, after being ashed with sulfuric and nitric acids, is treated with ferric chloride to remove the phosphate, and with ammonium acetate to remove the excess iron (as the basic acetate). The filtrate from these operations contains the bases, which are finally obtained as sulfates, free from interfering substances, by ignition with sulfuric acid and then with

ammonium carbonate. The final residue is analyzed for sulfate by the benzidine method,[1] and the total quantity of 0.1 N base present calculated from the result of this analysis.

In connection with the removal of phosphate and excess iron, the approximate directions commonly given in text-books of analytical chemistry for the adjustment of acidity, while adequate for use in the older gravimetric methods, are not sufficiently precise with the small quantities used in the present method, where more exact neutralization is required to prevent significant amounts of alkaline earth phosphates from being carried down with the iron precipitate.

Since the removal of phosphate proceeds more smoothly when the amount to be removed is known beforehand, a preliminary determination of phosphate is assumed in the detailed description following. This information can now be very readily obtained by Bell and Doisy's colorimetric method[2] with more than sufficient accuracy for the purpose. As an alternative, ferric chloride may be added until no further precipitate appears, but the presence of a large amount of basic ferric acetate makes the subsequent filtration much slower, and is likely to result in the consumption of more time than would be required for the phosphate determination.

Two variations of the method will be described. In the first, which is probably slightly more accurate, the iron precipitate is washed free from the desired bases with hot ammonium acetate solution, the filtrate cooled and diluted to a definite volume, and the analysis completed with an aliquot. This is time-consuming when many determinations must be made, and for that reason a much more rapid modification has been devised. In this case, the volume of the hot solution, after the addition of ferric chloride and ammonium acetate, is adjusted to 10 cc., and then cold water is added to make the total volume 25 cc. (and the temperature, therefore, about 50°C.). The solution can then be mixed, and the filtration completed in about 2 minutes. This procedure is, of course, necessitated by the fact that the basic ferric acetate would redissolve if the solution were allowed to become cold.

[1] Fiske, C. H., *J. Biol. Chem.*, 1921, xlvii, 59.

[2] Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.

The adjustment of volume at a temperature considerably above that of the room is not a serious matter, since a correction can easily be applied. The only real cause for concern lies in the possibility of concentration changes during the filtration, due to the escape of water vapor. By working under uniform conditions, and taking certain precautions, this error can be diminished to less than 0.5 per cent.

Removal of Phosphate.

First Method.—Measure into a large lipped Pyrex test-tube (200 by 20 mm.) a sample of urine containing an amount of chloride equivalent preferably to between ˙10 and 25 mg. of NaCl, but not more than 5 mg. of inorganic phosphorus. Add 1 cc. of 4 N sulfuric acid and 0.5 cc. of concentrated nitric acid, and boil down until white fumes appear. If the residue does not soon become colorless after this stage has been reached, cool slightly, add a few drops more of nitric acid, and continue the ·heating. When the remaining drop of sulfuric acid has become clear and colorless, let cool for a few minutes, and add 10 cc. of water and a drop of a saturated alcoholic solution of methyl red. Neutralize with powdered ammonium carbonate until the color of the indicator just begins to change, and restore the pink color by adding 4 N sulfuric acid, 1 drop at a time. Heat to boiling, and continue adding sulfuric acid in the same manner until 1 drop makes the solution faintly but unmistakably pink. Add a 10.5 per cent solution of ferric chloride ($FeCl_3 \cdot 6H_2O$) in 0.1 N hydrochloric acid in the proportion of 0.1 cc. for each mg. of inorganic phosphorus present in the original sample of urine,[3] shake, and run in 1 cc. of a 5 per cent solution of ammonium

[3] The ferric chloride solution may be delivered from an ordinary 1 cc. pipette, and measured with sufficient accuracy by counting the drops. The amount prescribed is about 20 per cent in excess, which should be enough to take care of any phosphate derived from organic phosphorus compounds in the original urine. To be perfectly safe, the filtrate should be tested for phosphate with the reagents of Bell and Doisy.[2] 5 cc. of the filtrate, when treated with 1 cc. of the molybdate solution, 1 cc. of hydroquinone solution, and 2 cc. of the carbonate-sulfite reagent, should give a color distinctly less than that obtained with 5 cc. of a phosphate solution, containing 0.005 mg. of phosphorus, treated in the same way.

acetate. Heat again to boiling. Filter on a 9 cm. *ashless* paper[4] into a 50 cc. volumetric flask, and wash with five 8 cc. portions of boiling 0.5 per cent ammonium acetate solution. Cool under the tap, make up to the mark, and mix.

Second Method.—Ash as described above, but add only 2 cc. of water to the cooled residue. Transfer to a test-tube marked at 10 and at 25 cc.,[5] rinsing three times with 2 cc. of water. The neutralization with ammonium carbonate and the precipitation with ferric chloride and ammonium acetate are conducted exactly as described under the first method. The hot solution at this stage should have a volume of 10 or 11 cc. Have ready a dry funnel fitted with a 9 cm. *ashless* paper,[4] and a dry 25 cc. Erlenmeyer flask. To the contents of the test-tube, which should be at the boiling point, add cold water (room temperature) to the 25 cc. mark. Immediately close the mouth of the test-tube with a clean, dry rubber stopper, invert two or three times, and filter without delay into the small Erlenmeyer flask, keeping the paper nearly filled as long as possible, and collecting only about 20 cc. of filtrate. The paper should fit the funnel snugly, and the entire filtration must not, in any event, occupy more than 2 minutes.[6] Stopper the receiving flask at once, and let cool (or cool under the tap if the analysis is to be completed immediately). The filtrate may contain a trace of iron, but this does no harm, since ferric sulfate decomposes on ignition. Larger quantities of iron would interfere mechanically.

[4] Nothing but the best quantitative paper will give dependable results. One determination with Schleicher and Schüll No. 604 filter paper on 2.5 cc. of 0.1 M KCl took 0.535 cc. of 0.1 N NaOH for an aliquot amounting to one-fifth of the total. This, calculated to the basis of the entire sample, gives 2.675 cc. of 0.1 N base, instead of 2.500 cc.

[5] The tubes used as receivers in the Folin-Wu urea method are suitable (Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 96). The 25 cc. mark must, of course, be accurate; that at 10 cc. need be only approximate and can be made with a glass pencil if necessary.

[6] 10 cc. of N sulfuric acid were heated to boiling, diluted to 25 cc. with cold water exactly as described, and filtered through a funnel plugged with paper so that 3 minutes were required for the filtration of 20 cc. The filtrate, after cooling, proved to be 0.4055 N, or, after applying the correction for temperature (1 per cent), 0.4015 N, instead of the theoretical 0.4 N. The error incident to evaporation was, therefore, only 0.4 per cent, although the filtration took half again as long as prescribed in the method.

Ignition and Sulfate Determination.

Transfer to a small platinum dish (about 5 cm. in diameter) an amount of the filtrate corresponding with the chloride equivalent of about 2.5 mg. of NaCl in the original urine,[7] add 1 cc. of 4 N sulfuric acid, and evaporate on the water bath until nearly dry. Place the dish on a metal triangle, and heat, cautiously at first, over a micro burner, gradually raising the flame until fumes have ceased to come off. Let cool, sprinkle over the residue a small amount of powdered ammonium carbonate, and ignite again, finally raising the flame to its maximum and moving the triangle about until each part of the dish has been momentarily subjected to a dull red heat. When the dish has cooled, add 2 cc. of water. Agitate until the residue has dissolved, using a rubber-tipped rod to assist in its solution if necessary. Transfer the contents of the dish to a large lipped Pyrex test-tube (200 by 20 mm.),[8] and rinse four times with 2 cc. of water. Add 2 cc. of benzidine reagent,[9] and, 2 minutes later, 4 cc. of 95 per cent acetone. After waiting 5 minutes for the completion of the precipitation, filter through paper pulp in the special filtration tube recently described,[1] wash three times with 1 cc. of 95 per cent acetone and once with 5 cc. With the aid of about 2 cc. of water and a nichrome wire, transfer the precipitate and mat through the bottom of the filtration tube into the test-tube in which the precipitation took place, and rinse off the wire with a few drops of water. Heat to boiling and add through the filtration tube (still suspended in the mouth of the test-tube) 2 drops of a 0.05 per cent aqueous solution of phenol red, and (from a

[7] For example, if the second method has been used with a sample of urine containing 12.5 mg. of NaCl, the proper amount of filtrate is one-fifth of the total, or 5 cc.

[8] Large Pyrex test-tubes provided with satisfactory lips are more convenient than beakers for use in connection with the benzidine sulfate method. Beakers were recommended before[1] because properly lipped Pyrex tubes had not been obtained at that time, and tubes of thinner glass cannot be relied upon to stand the treatment to which they must be subjected. The lipped Pyrex tubes used in this work were obtained from the Macalaster-Bicknell Co., 28 Wendell St., Cambridge 38, Mass.

[9] Suspend 4 gm. of benzidine in about 150 cc. of water in a 250 cc. volumetric flask. Add 50 cc. of N hydrochloric acid, shake until dissolved, and make up to volume. Filter if necessary.

micro burette) about 0.3 cc. of 0.1 N sodium hydroxide.[10]   Rinse down the sides of the filtration tube with about 3 cc. of water from a wash bottle, boil until steam escapes from the mouth of the test-tube, rinse again with about 2 cc. of water, remove the filtration tube, and continue the titration until 0.005 cc. of 0.1 N alkali produces a distinct pink color that does not change on further boiling.

The volume of alkali used gives, without further calculation, the amount of 0.1 N non-volatile base in the sample of filtrate analyzed in case the first method has been followed in removing the phosphate.   With the second method, a correction of 1 per cent must be subtracted to compensate for the contraction in volume on cooling the filtrate from 50° to room temperature.

Since reagents that give a wholly inappreciable blank appear to be easily obtainable, there is no reason for being satisfied with anything less good, for the necessity of correcting each determination for impurities in the reagents not only diminishes the accuracy of the method, but may also considerably complicate the calculation, inasmuch as some of the reagents are used in very variable amounts.   In order to test them, all that is required is a "determination" (by the second method) on the reagents alone (including 0.5 cc. of the ferric chloride solution).   5 cc. of the filtrate from the iron precipitate should be ignited with sulfuric acid and ammonium carbonate in the manner described, and 2 cc. of benzidine reagent introduced into the platinum dish containing the residue.   The absence of any precipitate of benzidine sulfate (after pouring the contents into a test-tube) is a very sensitive test, and shows that no significant amount of interfering impurity is present in the reagents.

Analysis of Mixed Salt Solution.

After sufficient preliminary work with potassium chloride solutions had been done to determine the most suitable conditions, the method was tested with a solution containing equivalent amounts of sodium, potassium, calcium, and magnesium,[11] with

[10] More dilute alkali may be substituted if desired but the amount of alkali that will be required is large enough to permit the use of a 0.1 N solution.

[11] Urine ordinarily contains much smaller proportions of alkaline earths than this, and they are the only bases of the four that are likely to cause trouble for specific reasons (by being carried down with the iron precipitate).

and without the addition of phosphate (as $NH_4H_2PO_4$). The following materials were used.

Sodium Chloride.—Recrystallized by saturating an aqueous solution with hydrochloric acid gas. Dried, and heated to incipient fusion.

Potassium Chloride.—Kahlbaum's (zur Analyse).

Calcium Sulfate.—Prepared by mixing equal volumes of 20 per cent calcium chloride and 20 per cent sulfuric acid. Filtered, washed, dried, and ignited to constant weight.

Magnesium Sulfate.—Prepared from Merck's "Reagent" magnesium oxide and sulfuric acid. Recrystallized, dried, and ignited to constant weight.

Monoammonium Phosphate.—By recrystallization of a Baker and Adamson preparation (wrongly labelled $(NH_4)_2HPO_4$). The final product was free from non-volatile bases.

The purity of the sodium and potassium chlorides was further checked by chloride determinations, and that of the calcium and magnesium sulfates by sulfate determinations.

A solution of each of these salts was prepared, equivalent to a 0.1 N solution of base (the calcium sulfate being dissolved in 0.5 N hydrochloric acid), and a mixture made of equal volumes of the four solutions. The results obtained with this mixture by the two methods are shown in Table I.

TABLE I.

Determination of Total Base in Solution Containing 0.1 M Na, 0.1 M K, 0.05 M Ca, and 0.05 M Mg.

Solution used.	P added.	First method			Second method.			
		Filtrate analyzed.*	0.1 N base.		Filtrate analyzed.†	0.1 N base.		
			Found.	Present.		Found.	Corrected.‡	Present.
cc.	*mg.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
10	0	5	1.005	1.000	3	1.210	1.200	1.200
10	5	5	1.005	1.000	3	1.205	1.195	1.200
					1	0.410	0.405	0.400
5	0	10	1.000	1.000	5	1.015	1.005	1.000
5	5	10	0.995	1.000	5	1.015	1.005	1.000
5	0	5	0.505	0.500				
5	5	5	0.505	0.500				
2	0	10	0.395	0.400	5	0.400	0.395	0.400
2	5	10	0.400	0.400	5	0.405	0.400	0.400

* Total volume of filtrate, 50 cc.

† Total volume of filtrate, 25 cc.

‡ By subtracting 1 per cent. All figures are given to the nearest 0.005 cc.

FAT-SOLUBLE VITAMINE.

X. FURTHER OBSERVATIONS ON THE OCCURRENCE OF THE FAT-SOLUBLE VITAMINE WITH YELLOW PLANT PIGMENTS.*

By H. STEENBOCK AND MARIANA T. SELL.

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When in 1919 (1) the senior author called attention to the apparent intimate association in the occurrence of the fat-soluble vitamine and yellow pigmentation it was stated that the data would be presented in detail as the evidence secured might warrant. In the meantime advantage has been taken of this observation to study the nature of the vitamine, assuming that such association was of more than mere physiologic significance. While this was in progress the publication of our data has been delayed.

Originally we were led to surmise a possible intimate relationship between pigment and fat-soluble vitamine on the premises that the leafy parts of plants, and carrots and sweet potatoes—all containing large amounts of yellow pigment—were also rich in the vitamine. Many fats low in pigment had already been found correspondingly poor in vitamine. Furthermore, tubers and roots such as the potato, dasheen, mangel, sugar beet, and red beet—in contrast to carrots and sweet potatoes—all poor in yellow pigment content, were also found poor in vitamine 'or even entirely free from it.

These conditions were taken as merely suggestive as even to the casual observer many instances would immediately occur which indicate absolute non-conformity with the above conditions. Such is especially true with many fats, some of them

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being highly pigmented and yet impotent as a source of vitamine. Also in many plant materials we early noted a lack of proportionality between vitamine and yellow pigment content.

In animal husbandry circles there has always prevailed a vague suspicion that the yellow and white varieties of Indian corn were not entirely equivalent in feeding value. Such was also our inference when we found that the only instances of ophthalmia in our stock colony of rats were observed when we substituted white corn for yellow corn in their rations. Apparently our animals had been kept on a ration dangerously low in fat-soluble vitamine which fact did not, however, become immediately evident until this substitution was made. Carefully planned feeding trials with many different varieties of maize have now left no question but that yellow maize as compared with the white varieties is immeasurably superior in fat-soluble vitamine content (2).

Peas have been graded variously as to their fat-soluble vitamine content. Chick and Delf (3) found both dry and germinated peas deficient. Coward and Drummond (4) credit them with more or less fat-soluble vitamine activity. McCollum and co-workers (5) obtained good growth on 87.5 per cent of peas but state that the addition of butter fat would have improved the ration for reproduction. We have found it possible to harmonize these points of view as upon analyzing different varieties we (6) found that they differed tremendously in their vitamine content; those richest in vitamine were again found highest in yellow pigment content.

Outside of the data reported from the author's laboratory (7) unfortunately there appear to be none available on the vitamine content of sweet potatoes and carrots. However, as our original observations have now been repeatedly confirmed in successive years on different samples, there appears to be no question but that the ordinary yellow and reddish yellow varieties are abundantly supplied with it. In fact our most recent data indicate an occasional occurrence of even larger amounts of vitamine than we had originally surmised to be present. With the association of vitamine with yellow pigment established in the previously mentioned plant materials we have now extended our investigations to include the white as well as the pigmented varieties

of carrots and sweet potatoes. Our observations here are limited, but so far, the non-pigmented varieties were again found very low in vitamine.

Leafy materials have repeatedly been shown to be well supplied with the fat-soluble vitamine. We have here also availed ourselves of an opportunity to demonstrate the association of vitamine with yellow pigments by feeding green and etiolated cabbage leaves, leaves respectively rich and poor in yellow pigments.

EXPERIMENTAL.

All our demonstrations of fat-soluble vitamine activity were made with rats of mixed black and albino ancestry. These were taken at an age of 3 to $4\frac{1}{2}$ weeks at the corresponding weights of 40 to 70 gm. and put on a basal ration of salts, casein, dextrinized starch, and a source of water-soluble vitamine, with which the unknown to be tested was incorporated.

The salts used were respectively Salt Mixture 32, the composition of which has already been given (7), and Salt Mixture 38 which represented a mixture of 3 parts of Salt Mixture 32 with 1 part of Salt Mixture 35 (7).

The casein was a high grade of commercial casein purified by washing for a week with two changes of dilute acetic acid daily. It was finally washed with distilled water, then dried and baked in shallow pans at 95° for at least 72 hours.

The dextrinized starch was prepared by barely moistening cornstarch with a 0.1 per cent solution of citric acid and then autoclaving it for 2 to 3 hours at 15 pounds steam pressure. After drying at 80 to 85° for at least a week it was ground to a fine powder.

The water-soluble vitamine was incorporated with different materials. In some of the experiments we used 40 per cent of white corn. This introduced a superabundance of water-soluble vitamine, but with it were also introduced small amounts of the fat-soluble vitamine or some other growth constituent, because with it present in the ration usually better growth is secured than with water-soluble vitamine from other sources. For comparative purposes its use was, however, entirely permissible. In some instances 2 per cent of dried yeast was used as the carrier

of the water-soluble vitamine and again in other cases 3 per cent of ether-extracted wheat embryo or a larger amount of the alcohol extract of ether-extracted wheat embryo evaporated on and made up to the original weight of the wheat embryo. This latter preparation we have used repeatedly in our laboratory and for the sake of convenience have given it the name of dextrin wheat embryo.

The sweet potatoes were sliced and dried at room temperature in an air current or else, as noted, were dried in an oven at approximately 80 to 85°. As the circulation of air in this oven was very limited, the potatoes were well steamed by their own moisture content before desiccation had ensued to any noticeable degree.

The carrots were trimmed to remove the crown in order to avoid contamination of the root with the attached leaf stems. They were shredded in a power-mill and dried at room temperature in an air current.

Results with Sweet Potatoes.

In Chart 1 are shown the growth curves obtained when 15 per cent of oven-dried sweet potatoes furnished the sole source of fat-soluble vitamine. The three varieties on which all our tests were conducted are represented in these trials. The Triumph variety was a root with flesh of a creamy white color, Little Stem Jersey had flesh of a light yellow color, and Nancy Hall flesh of an intense yellow color.

As is seen in Chart 1 in Lot 932, failure of growth was complete in all individuals on the Triumph samples at the end of 7 weeks. By this time the eyes of Rats 3722 and 3725 were edematous and at times completely closed—symptoms entirely characteristic in the reaction of the mammal to a deficiency of the fat-soluble vitamine. When this deficiency was removed by the introduction of butter fat, the eyes promptly cleared up and growth was resumed.

Individuals in Lots 820 and 821 on the yellow pigmented roots grew at what might be called the normal rate. Though young were produced none were reared, which was to be expected as lactation is far from normal on such rations even when their fat-soluble vitamine content is more than sufficient. Further data are shown in Chart 2.
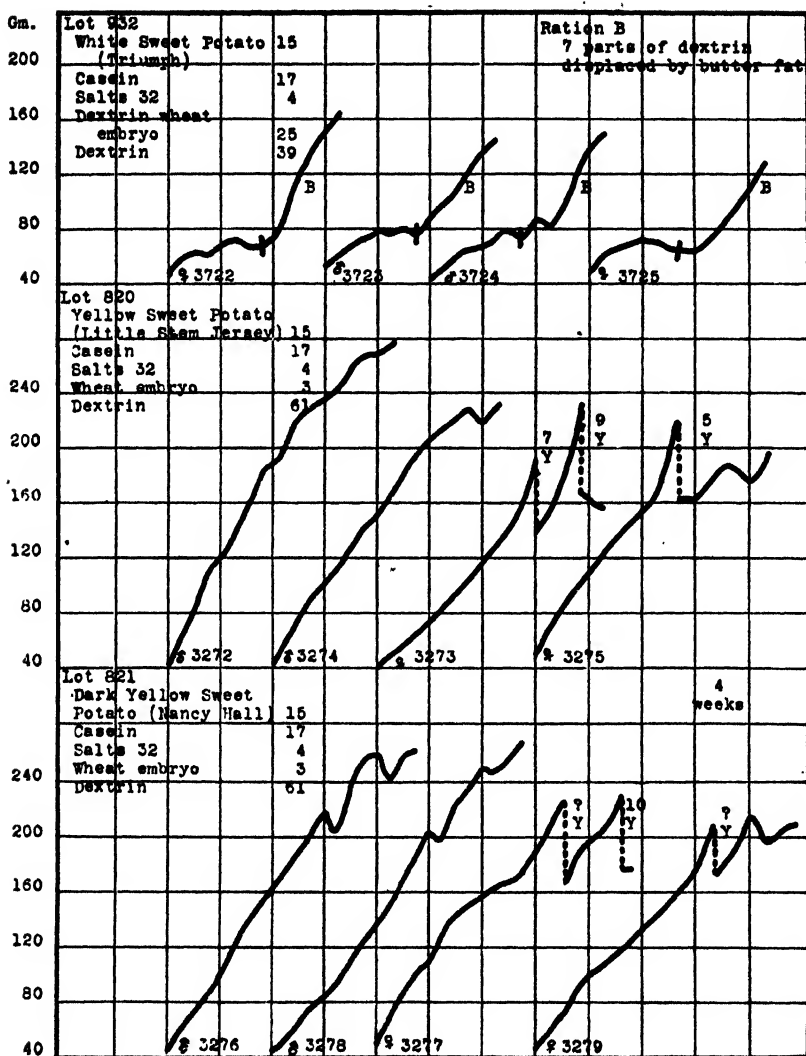
CHART 1.

As in the case of the experiments shown in Chart 1 complete failure again resulted on the Triumph sweet potatoes which as well as the other varieties were air-dried. This was to be expected as they were fed at a lower level, 5 per cent as compared
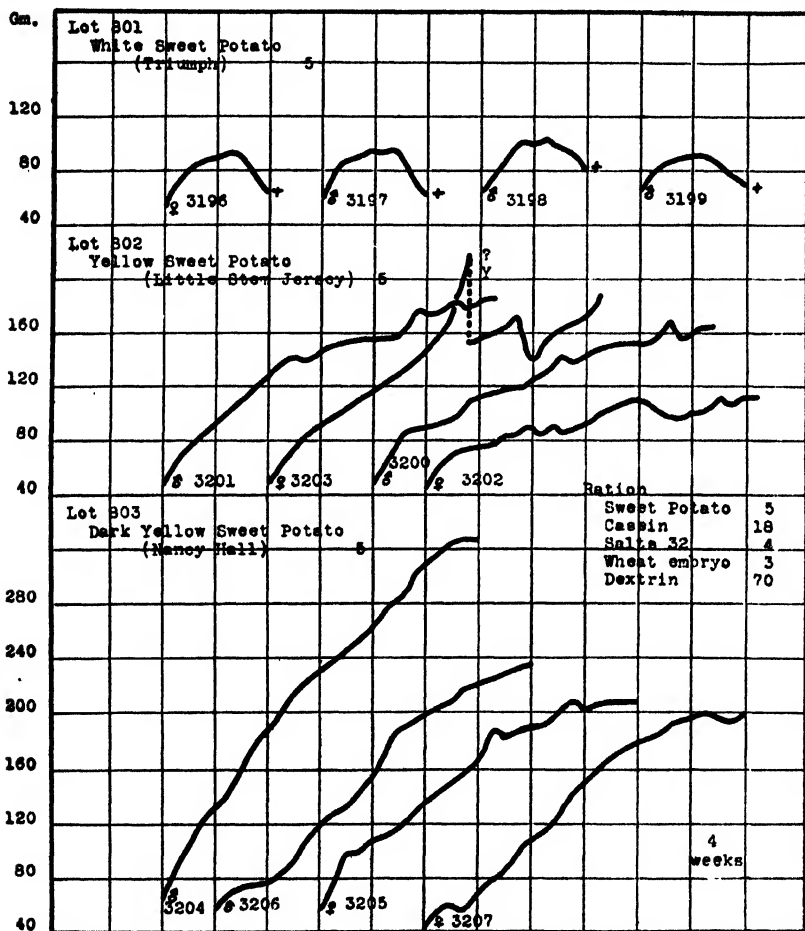
CHART 2.

with 15 per cent formerly. Even at this level, however, about half normal growth was observed on the yellow sweet potatoes and about three-quarters normal growth on the deep yellow sweet potatoes. This indicates that the fat-soluble vitamine must be found in considerable amount in these varieties.

In Lot 937, Chart 3, are shown data indicating that the Triumph sweet potato is not entirely devoid of fat-soluble vitamine. When fed at a 25 per cent level a considerable amount of growth was obtained but after a few months complete failure resulted. Rats 3743 and 3744 both came down with a severe ophthalmia which was promptly cured upon the addition of
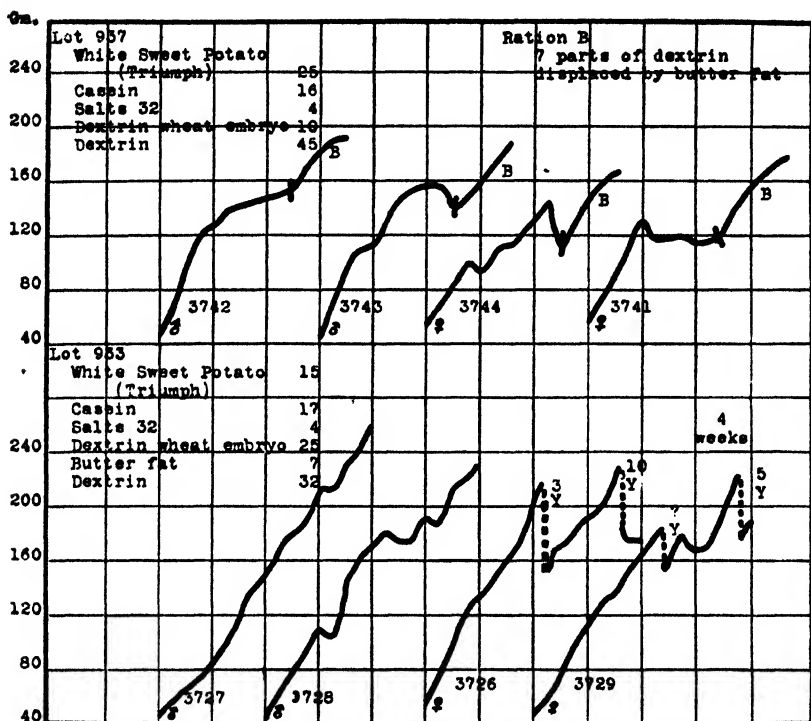
CHART 3.

butter fat. With Lot 933, Chart 3, it is shown that the sweet potato does not contain anything sufficiently injurious to the well being of the rat to prevent growth if fed in a ration not deficient in necessary constituents.

Results with Carrots.

The feeding trials with carrots were carried out on two different samples of a white variety, one of a yellow variety, and one of a reddish yellow variety. The white carrots were distinctly of the

type used for stock feeding. One sample, the last fed, was of very luxuriant growth, approximately one-half of the root having been exposed during its growth to the light as indicated by the fact that the upper part was more or less pigmented with chlorophyll. This was not true of the other varieties although the yellow variety also carried some chlorophyll which by comparison with the sample of white carrots were mere traces only. The variation in chlorophyll content suggested the possibility of difference in yellow pigmentation and also fat-soluble vitamine content. To determine the latter, the roots of all three varieties were cut approximately in half, giving a top and bottom portion. Each was fed separately, incorporated in fat-soluble vitamine-free basal rations. As these trials were not all carried out at the same time but were run for comparative purposes in correlation with other experiments, it has happened that different basal

TABLE I.

Rations.	I	II	III
Casein............................	18	14	18
Salts.............................	4 (32)	4 (38)	4 (32)
White corn........................	0	40	0
Wheat embryo......................	3	0	0
Yeast.............................	0	0	2
Dextrin to........................	100	100	100

rations were used. These had the composition shown in Table I.

They are all low in fat-soluble vitamine, but other things being equal, we have indications that they range in their content of fat-soluble vitamine—to the extent that it can be determined—in the order of II, I, III. It so happened then, that with white carrots where the poorest growth was obtained, basal rations of the highest vitamine content were employed. The results of these feeding trials are shown in Table II. In it has also been incorporated data obtained on the fat-soluble vitamine content of the carrot leaves.

The data again indicate the inferiority in fat-soluble vitamine content of the non-pigmented roots. To ascertain to what extent pigment accompanied vitamine, analyses of the top and bottom portions of the white carrots were made for pigment as well as for vitamine. To this end 15 gm. of material were extracted

with alcohol for 6 hours in a Soxhlet. The alcohol was evaporated off from the extract to a small volume and the residue saponified over night with alcoholic potash prepared with the usual precautions. After dilution with water the pigments were shaken out with petroleum ether, concentrated to a small volume made up to volume, and compared in the Duboscq colorimeter. Approximately twice as much pigment was found in the tops as in the bottoms. This, as shown in the table, also harmonizes with their vitamine content.

Results with Cabbage Leaves.

For comparative results with cabbage there were used, on the one hand, all the etiolated leaves—entirely free from chlorophyll—as obtained from fresh heads of cabbage, and on the other hand, the small green leaves obtained from cabbage plants which at the end of the growing season had failed to head. This selection was made to avoid possible differences in vitamine content incident to age and other factors. The material was dried at room temperature and fed pulverized in a basal ration of casein, 18; wheat embryo, 3; Salts 32, 4; and dextrin to 100. The data as obtained are presented graphically in Chart 4.

The white cabbage leaves were found considerably inferior to the green in vitamine content.

Rats 3078 and 3079 in Lot 771 and Rats 3297 and 3299 in Lot 826 on the white cabbage at the 5 and 10 per cent levels came down with an ophthalmia and Rat 3296 of Lot 826 with a respiratory infection. This, with the inferior growth and sudden ultimate failure of most of the animals stands in marked contrast with the results obtained on green cabbage where the growth, though slow on the 5 per cent level, was nevertheless continued and with maturity accompanied by reproduction. Our results on feeding the white cabbage stand in marked contrast to those obtained by Coward and Drummond (8) who report practically no growth on a supplementary intake of 1.5 gm. of fresh cabbage daily while on such an amount of green cabbage they obtained fair growth. Unfortunately, however, in their work they fail to state the source of their green leaves which we accordingly assume to have been the outer leaves as they are most readily available. This does not make their data exactly comparable to

TABLE II.

Lot.	Carrots.	Percentage.	Ration.	Rat No.	Sex.	0 gm.	2 gm.	4 gm.	6 gm.	8 gm.	10 gm.	12 gm.	14 gm.	16 gm.	18 gm.	20 gm.	22 gm.	Remarks.
764	White.	15	1	3048	♀	55	84	107	112	105								Rats 3048 and 3050 had both eyes inflamed at the end of the 8th week. Rat 3051 had respiratory trouble.
				3049	♀	52	67	81	76	67								
				3050	♂	65	87	105	100	86								
				3051	♂	50	82	111	128	115								
1229	White tops.	7.5	2	4861	♂	52	82	106	140	153	110	+						Rat 4861 had inflamed eyes at the end of the 9th week. The others later contracted respiratory trouble.
				4862	♀	47	79	102	140	160	170	185	198	190	180	180	+	
				4863	♂	52	77	95	112	112	110	103	90	75				
				4864	♀	48	82	111	145	167	175	178	180	179	182	175	131+	
1228	White bottoms.	7.5	2	4857	♂	48	71	86	92	+								Rat 4857 died at the end of the 7th week, symptoms not noted. Rats 4858 and 4859 had inflamed eyes at the end of the 7th week and Rat 4860 at the end of the 8th week.
				4858	♀	48	78	98	109	117	90	+						
				4859	♂	52	84	105	117	117	108	83	+					
				4860	♀	52	87	102	122	130	128	135	+					
1377	Yellow tops.	3	3	4379	♂	63	103	138	177	209	211	235	247	257	260			Rats in good condition.
				4380	♀	50	77	110	135	165	170	180	197	190	185			
				4381	♂	62	88	117	170	205	220	245	262	275	260			
				4382	♂	45	72	107	145	172	186	202	202	215	225			
1376	Yellow bottoms.	3	3	4375	♂	52	77	113	147	171	200	208	218	228	238			Rats in good condition except Rat 4378, which died from unknown causes.
				4376	♀	61	83	110	122	131	147	165	192	195	202			
				4377	♂	65	108	138	165	181	202	220	230	247	257			
				4378	♂	43	68	98	107	+								

				Rat	Sex											Remarks
1379	Red tops.	3	3	4387	♂	45	88	130	165	182	200	203	212	230	230	Rats in good condition. Rat 4388 had young during the 10th week.
				4388	♀	66	100	120	140	165	200	190	202	213	210	
				4389	♂	45	67	103	137	157	182	187	195	213	220	
				4390	♂	43	70	113	145	160	180	188	198	208	207	
1378	Red bottoms.	3	3	4383	♂	66	115	172	205	228	264	270	278	283	292	Rats in good condition.
				4384	♀	62	92	118	135	145	182	187	211	197	190	
				4385	♂	48	80	140	175	190	215	217	228	238	240	
				4386	♀	45	73	95	115	138	152	155	175	168	163	
1335	White carrot leaves.	2	2	4218	♂	55	100	152	195	228	255	280	290			Rats in excellent condition. Rat 4319 had two litters of young, one during the 9th and one during 13th week.
				4219	♀	52	80	112	145	211	195	255	220			
				4220	♂	49	80	118	162	208	235	250	269			
				4221	♂	54	85	125	167	193	225	250	270			
1336	Yellow carrot leaves.	2	2	4222	♂	55	95	140	177	211	230	257	280			Rats in good condition. Rats 4222 and 4225 had suspicious looking eyes, probably trauma, certainly not an ophthalmia due to deficient fat-soluble vitamine.
				4223	♂	59	92	137	182	213	228	252	258			
				4224	♂	49	71	100	110	176	182	208	220			
				4225	♂	55	?	135	178	215	240	257	273			
1337	Red carrot leaves.	2	2	4226	♂	50	87	128	186	217	250	255	258			Rats all excellent.
				4227	♂	68	100	150	185	215	240	250	261			
				4228	♂	52	90	147	200	247	280	300	306			
				4229	♀	66	90	122	150	175	203	195	197			
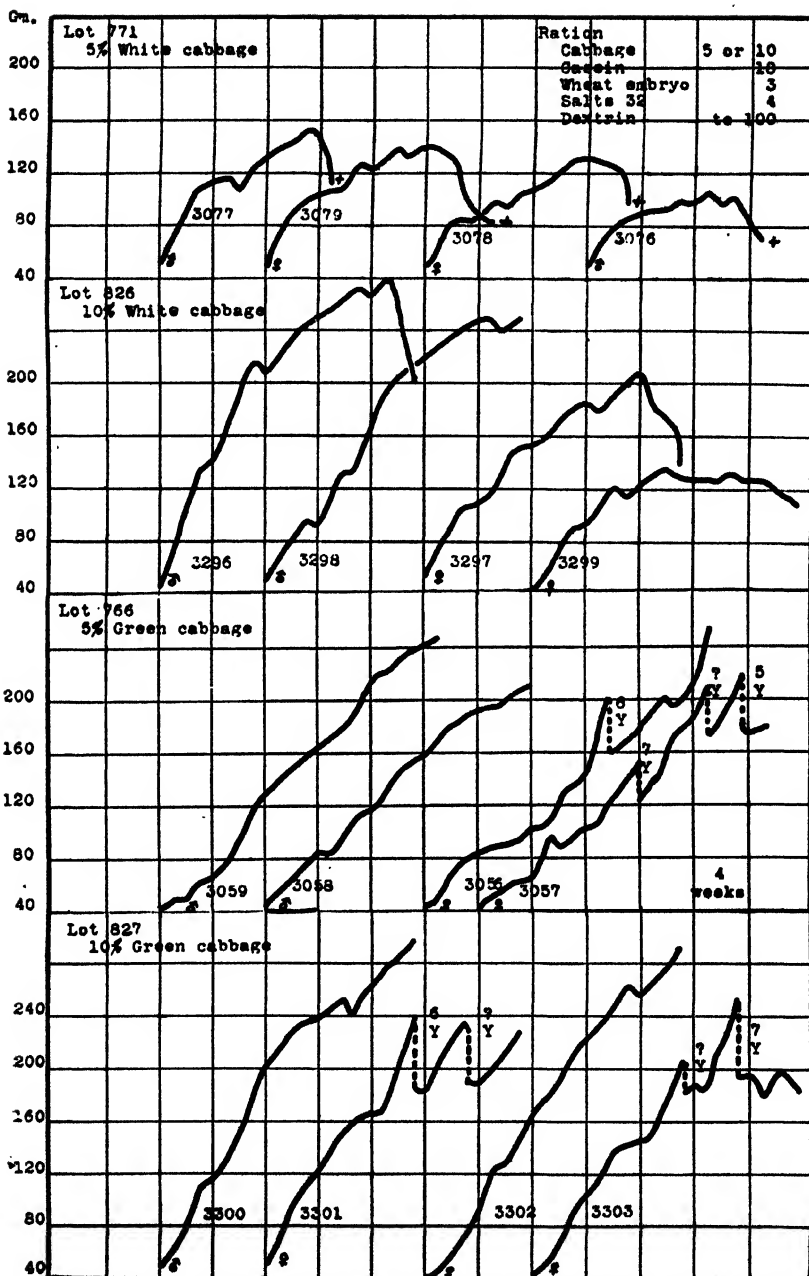
CHART 4.

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ours, nevertheless, it is interesting to note that they likewise report a considerable vitamine content in the green material. Delf (9) has also stated that the inner white leaves of cabbage do not contain "growth-promoting vitamines." McCollum, Simmonds, and Pitz (10), Osborne and Mendel (11), and the author and coworkers (12), have all reported the presence of the fat-soluble vitamine though in lesser amounts than in other leafy materials. We believe it safe to assume that the fat-soluble vitamine is generally present in more than the minimal demonstrable amounts in white cabbage and that Coward and Drummond failed to demonstrate it because the level at which it was fed was too low to bring out its presence. As Delf's data have not been published in detail we are not able to explain the results obtained unless it is to be assumed that some varieties contain much more of it than others.

A sample of the green and white leaves analyzed for pigment content by the method used with carrots, revealed a tenfold greater pigment content with the green leaves than with the white.

CONCLUSIONS.

Data are presented which further emphasize the fact that the fat-soluble vitamine often occurs most prominently where there are found the largest amounts of certain yellow pigments. White sweet potatoes and white carrots were found to contain little fat-soluble vitamine which stands in marked contrast to our observations on the yellow pigmented varieties. The tops of white carrot roots, slightly pigmented with chlorophyll and containing a small amount of yellow pigment were found richer in fat-soluble vitamine than the bottoms containing only one-half as much pigment. Green cabbage leaves taken from the heart of cabbage plants which failed to "head" were found much richer in fat-soluble vitamine than white cabbage leaves in the head. The latter contained only one-tenth as much yellow pigment.

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WATER-SOLUBLE B AND BIOS IN YEAST GROWTH.

By ELLIS I. FULMER and VICTOR E. NELSON.

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During the past 2 years there has been considerable discussion relative to water-soluble B as a possible stimulant of yeast growth. Until recently the prevailing opinion was to the effect that the yeast growth stimulant, bios, and water-soluble B are identical. In fact, the stimulative effect upon the growth of yeast of extracts of materials was proposed (1) as a quantitative measure of the water-soluble B content of the materials.

In recent articles[1] by Fulmer, Nelson, and Sherwood (2), experimental data were presented which showed that the method of measuring vitamine content by yeast growth stimulation was of no value for the following reasons: (*a*) the relative potencies of two materials as yeast growth stimulants cannot be arrived at on the basis of the stimulative effect of the extracts from equal weights of materials; (*b*) treatment with alkali does not impair the stimulative effect of the alcoholic extracts, a treatment known to destroy water-soluble B; (*c*) alcoholic extracts of alfalfa and wheat embryo contain sufficient nitrogenous and mineral nutriment for the growth of yeast; (*d*) a medium of known composition has been developed, namely Medium F, which promotes the growth of yeast without vitamine. The addition of alcoholic extracts of wheat embryo or alfalfa does not improve this medium. Yeast has been growing in the above medium for nearly 2 years being subcultured at least 300 times, each time with a dilution of 1:50. In a further communication Nelson, Fulmer, and Cessna (3) presented data showing the synthesis of water-soluble B by yeast.

In a recent paper by Eddy, Heft, Stevenson, and Johnson (4) certain exceptions are taken to the work mentioned above and,

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[1] Reports on which were read at the 1920 spring meeting of the American Chemical Society, St. Louis.

furthermore, some interpretations have been placed upon that work which we feel to be unjustified.   For instance, they state:

"In this connection they [Fulmer, Nelson, and Sherwood] propose a formula for the culture medium (Medium F) which they believe will produce maximum stimulation to yeast growth and which will not be improved or altered by the addition of extracts of organic substances unless the latter disturb the optimum salt concentrations of the medium."

We have not maintained that Medium F will produce maximum stimulation of yeast growth.   If such were the case it would imply that Medium F is as good a medium as beer-wort.   What we said and implied was that Medium F is the best synthetic medium for the growth of yeast which has been developed up to the present time and that it is the best possible medium which can be made from the constituents employed.   Furthermore, we do not maintain that Medium F cannot be improved by means of organic extracts.   Since Medium F is not as good a medium as is beer-wort it would necessarily follow that certain organic extracts would improve Medium F.   We maintain only that the addition of *alcoholic* extracts from alfalfa and wheat embryo, containing water-soluble B, will not improve the medium.   The same authors go on to say: "In brief, they [Fulmer, Nelson, and Sherwood] imply that 'bios' is simply a matter of salt concentration."   In a recent communication Funk and Dubin (5) apparently are unable completely to substantiate the work of Fulmer, Nelson, and Sherwood, and agree with the conclusions of Eddy, Heft, Stevenson, and Johnson (4).   Moreover, they maintain that Medium F is not as good as Nägeli's solution for the growth of yeast, a conclusion at variance with that of Eddy, Heft, Stevenson, and Johnson (4) who say that "Medium F is superior to Nägeli solution as a basal medium."   Furthermore Funk and Dubin (5) propose a new name for bios, namely vitamine D, a procedure which we believe to be wholly unnecessary and confusing.   The term bios has proved satisfactory and, moreover, the name vitamine D should be reserved for any new vitamine that may subsequently be found necessary for the growth and well being of the animal.

There seems to be a tendency to confuse the terms vitamine, bios, and auximone.   Vitamines are materials of unknown composition, which are necessary in the diet, other than fats, proteins,

carbohydrates, and mineral salts, for the growth and well being of the animal. Bios is the term originally applied by Wildiers (6) to designate substances of unknown composition which are necessary for the best growth of yeast. Auximone is a term originally used by Bottomley (7) to designate those substances of unknown composition believed to be necessary for the growth of plants. Any interchange of the above terms is in our present state of knowledge unjustified. While yeast will grow so satisfactorily in Medium F that addition of water-soluble B will not improve it, the addition of certain other extracts will improve the medium greatly. Our point was that bios and water-soluble B are not identical.

In the paper referred to (Eddy, Heft, Stevenson, and Johnson, 4) they agree with us on the first two of our claims; *i.e.*, that the relative stimulating potencies of two materials cannot be arrived at on an equal weight basis, and that alkali does not destroy the stimulant; as a result, at least temporarily, they discard the yeast growth stimulation method for the estimation of water-soluble B.

The authors do, however, take definite exception to our statement that medium F is not improved by the addition of extracts (alcoholic) of alfalfa and come to the conclusion "that while Medium F is superior to Nägeli solution as a basal medium it is still capable of stimulation by every concentration of the extract used."

It seemed advisable, in view of the above statement, to check our previous data. Dried alfalfa was extracted for 12 hours in a continuous extractor with 95 per cent alcohol (the method outlined in our previous papers, 2). Varying concentrations of the extract were added to Medium F which had the following composition: 100 cc. contained 0.188 gm. of ammonium chloride, 0.100 gm. of calcium chloride, 0.100 gm. of dipotassium phosphate, 0.04 gm. of precipitated calcium carbonate, 0.60 gm. of dextrin, and 10 gm. of cane-sugar. Of course, it is necessary when the dextrin is prepared by heating starch with citric acid to extract the acid with alcohol previous to its use in the medium. The flasks were inoculated with an initial count (when the count = 1, there are 250,000 cells per cubic centimeter) of about four, the yeast being taken from cultures which had been growing in

Medium F for 2 years. The cultures were incubated at 30°C. After 48 hours the yeast count was determined. The results are given in Table I, Column 1. By "dry equivalent" is meant the number of grams of dry alfalfa added as extract per 100 cc. of medium.

The addition of alcoholic extract of alfalfa did not improve Medium F, thus verifying our previous statement that not only is water-soluble B not necessary for the growth of yeast but also it is of no advantage in Medium F.

A study of the methods used by Eddy, Heft, Stevenson, and Johnson, revealed two points wherein their method differed from that used by us and which might account for their failure to

TABLE I.

(1) Dry equivalent.	(2) Alcoholic extract.	(3) Extract according to Eddy, Heft, Stevenson, and Johnson.
0	214	214
0.10	216	250
0.20	214	260
0.40	221	300
0.60	223	400
0.80	220	325
1.00	206	306
1.20	226	300
1.40	193	246
2.00	184	

check our results. First, there is the matter of temperature control. They used temperatures varying between 30–35°C. Medium F is optimum for 30°C. and for that temperature only. Secondly, the above authors did not use a 95 per cent alcoholic extract of alfalfa. Their method was as follows: "400 gm. of dried alfalfa were repeatedly extracted with distilled water, the filtered extracts combined . . . ." They sometimes prepared the extract by "diluting the water extract with 95 per cent alcohol to 40 per cent of the volume and filtering off the precipitated protein complex."

Extract of alfalfa was prepared according to the method outlined above. While the alcoholic (95 per cent) contained 0.20 gm. of dry material per 100 cc. the water extract contained 0.70 gm. of dissolved material per 100 cc.

Varying concentrations of the extract were added to Medium F in the same manner as outlined above for the 95 per cent alcohol extract. The results are tabulated in Table I, Column 3. The addition of the water extract did stimulate the growth of yeast in Medium F. This extract evidently contains, along with water-soluble B and other materials, bios, the yeast growth stimulant. The 95 per cent alcoholic extract contains water-soluble B but not bios.

We wish to thank Mr. H. W. Wright for aid in obtaining experimental data.

SUMMARY.

1. Data is presented in this paper which verify previous work to the effect that 95 per cent alcoholic extract of alfalfa does not improve Medium F for the growth of yeast.

2. Two explanations and supporting data are given why Eddy, Heft, Stevenson, and Johnson, and also Funk and Dubin, were unable to corroborate our results.

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6. Wildiers, E., *La Cellule*, 1901, xviii, 313.
7. Bottomley, W. B., *Proc. Roy. Soc. London, Series B*, 1915–17, lxxxix, 102, 481.

A REPLY TO FULMER, NELSON, AND SHERWOOD CONCERNING MEDIUM F.

By WALTER H. EDDY, H. L. HEFT, AND H. C. STEVENSON.

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Opportunity having been given us to examine the preceding paper prior to its publication we take this opportunity to present certain comment thereon.

In our original paper we were concerned solely with the question of whether the stimulatory action of vitamine B-containing extracts owe their stimulatory effect to the vitamine and whether the yeast test first suggested by Williams and later modified by several workers is a reliable index of the vitamine B content of an extract. In the two papers of Fulmer, Nelson, and Sherwood that were before us at the time[1] the claims bearing on this point were as follows: (1) Alcohol extracts of alfalfa and wheat embryo do stimulate yeast growth when added to synthetic media of the Williams' type. (2) Treatment of these extracts by alkali fails to reduce their stimulatory powers. (3) Alcohol extracts of alfalfa and wheat embryo fail to show stimulatory effect when added to a medium devised by the authors and called by them Medium F. The authors say: "From the above data it is evident that we have developed a medium, namely Medium F, composed of known constituents, which is not improved by the additions of vitamine containing extracts."

We reported in our paper substantial agreement with all except one of the claims of the authors (superiority of Medium F over the Williams' type, the negative effect of alkali upon the stimulatory extracts, and the impossibility of using the test in its present form as a test for vitamine content). We believed at the time that the authors had used alcohol extracts solely as a means of obtaining a vitamine B extract, after the method devised by

[1] Fulmer, E. I., Nelson, V. E. and Sherwood, F. F., *J. Am. Chem. Soc.*, 1921, xliii, 186, 191.

McCollum for extraction of that vitamine from the navy bean. We used a water extract for the same purpose, as it had been repeatedly shown by various investigators that 95 per cent alcohol is a relatively poor extractant of vitamine B. We had no idea at the time that water and alcohol extracts were to be considered as qualitatively different for the purpose in mind, *viz.* to determine the effect of vitamine B on the medium. We, therefore, believed our criticisms of the conclusions were a correct statement in view of the fact that our water extracts did stimulate Medium F.

In the present paper the authors repeat our work with water extract and confirm our results. They repeat their own work with alcohol extract and confirm their own results. They then criticize our interpretation, made on the above basis, and advance a new view-point, *viz.*, that water extracts both bios and vitamine B but alcohol extracts only vitamine B. Herein is a suggestion quite in accord with that of Funk, and forecast by Emmett, namely that the yeast stimulatory substance is something other than vitamine B and in the present paper admittedly not present in Medium F. They prefer to retain for this factor the term bios, Funk to call it vitamine D. Emmett declined to name it, but suggested it was unlike vitamine B in not being antineuritic. While such a view is quite possibly accurate, it has seemed to us desirable first completely to exclude vitamine B from the factors involved in the yeast growth and evidence to this effect seems at present definitely lacking. We have not yet had the opportunity to fully investigate the relative properties of alcohol and water extracts of alfalfa but we have prepared an alcohol extract of this substance after the manner of Fulmer and associates,[2] and used this in the concentrations given by the authors and in higher concentrations. In our incubations this time, our incubator was set at exactly 30°C. The results are given in Table I. We continued to use the Funk method of measurement as we believe it to be more accurate as a measure of total growth than their method.

The results make us sceptical as to the qualitative differences in alcohol and water extract as we again *get stimulation of Medium F and with the alcohol extract.* The stimulation is not as great as

[2] Fulmer, Nelson, and Sherwood,[1] 187.

with the water extract, which is quite in harmony with the view we had held, that alcohol is a poorer extractant of vitamine B than water. It is also to be noted that the results obtained with us which showed greatest stimulation are with concentrations much greater than those used by Fulmer and associates, which facts combined with what we believe to be greater accuracy in our measuring, could easily account for their failure to observe stimulation with the alcohol extract.

TABLE I.

Concentrations used by Fulmer, Nelson, and Sherwood. Dry equivalent in 100 cc.	Concentrations used by us in repeating Fulmer, Nelson, and Sherwood's work.	Growths obtained by us with an alcohol extract of alfalfa expressed in 1000ths of a cc. of yeast cells, Funk method. Duplicate determinations.			Growths obtained by us and reported in previous paper when water extract of alfalfa was used.		
		1	2	Average.	Dry equivalent expressed after manner of Fulmer, Nelson, and Sherwood.	Corresponding concentrations as expressed in Table III, Series C of our original paper.	Average of five determinations.
0.00	0.00	13	10	11	0.00	Control.	15
0.10	0.10	15	15	15			
0.20	0.20	10	15	12			
0.40	0.40	13	17	15			
0.60	0.60	15	16	15	0.60	0.015	19
0.80	0.80	15	13	14			
1.00	1.00	13	15	14	1.00	0.025	18
1.20	1.20	16	15	15			
1.40	1.40	17	22	19	1.40	0.035	19
2.00	2.00	16	17	16	1.80	0.045	19
	2.50	16	20	18	3.00	0.075	22
	5.00	16	18	17	5.00	0.125	25

We have no desire to develop controversy in this field. Our own results, including those accumulated since the publication of our paper, have only served to increase our distrust of the test as an accurate measure of vitamine B but they have also clearly indicated the need of very careful study to evaluate all the factors concerned in the response of the yeast cell and if these factors can be evaluated we believe that it may result in showing that vitamine B plays a part in the action and in perfecting the test for the detection of that factor.

THE HYPOBROMITE REACTION ON UREA.

By PAUL MENAUL.

(From the Oklahoma Agricultural Experiment Station, Stillwater.)

In the determination of urea in the urine of experimental animals a discrepancy in the results was noticed upon using the urease method of Folin and Youngburg[1] and the hypobromite method of Stehle,[2] the hypobromite method giving low results.

Two published articles,[3,4] were brought to my notice by Dr. C. T. Dowell of this Department, as bearing upon the subject. These articles declare the formation of dichloro urea and nitrogen trichloride in reactions between chlorine and urea. Only 93 to 96 per cent of the nitrogen being liberated in gaseous form, showing that this is not a stoichiometric reaction, which is also shown by Krogh,[5] who finds that in the reaction of sodium hypobromite on urea, that part of the nitrogen is set free, and that the rest is converted into oxides of nitrogen. With a variation in the concentration of the bromine, there is also a variation in the amount of gas liberated, a more dilute solution of bromine liberating more gas. The volume of nitrogen varied from 86.8 to 96.5 per cent of the nitrogen in the urea. By what law of physical chemistry should such an oxidation reaction be altered by vacuum?

The reaction between sodium hypobromite and urea is nearly instantaneous, and it is not apparent to the author why Stehle considers that in his method the reaction takes place *in vacuo*, for it is apparent that the reaction has proceeded for some time before any vacuum can be obtained.

In order to solve this question a number of determinations were made using pure urea oxalate. The apparatus and pipette were calibrated with mercury. The hypobromite solution was added

[1] Folin, O., and Youngburg, G. E., *J. Biol. Chem*, 1919, xxxviii, 111.

[2] Stehle, R. L., *J. Biol. Chem.*, 1921, xlvii, 13.

[3] Chattaway, F. D., *Chem. News*, 1908, xcviii, 285.

[4] Dowell, C. T., *J. Am. Chem. Soc.*, 1919, xli, 124.

[5] Krogh, M., *Z. physiol. Chem.*, 1913, lxxxiv, 379.

through the exit tube (of the Van Slyke apparatus) subjected to vacuum, the air expelled, then the urea solution and rinse water were added through the graduated cup, the air dissolved in them having been previously determined.

The following is a table of representative results obtained by following the procedure in the original article of Stehle, using also the strength of bromine recommended by Dehn[6] and Krogh.[5]

TABLE I.

Nitrogen in Urea Determined by the Following Methods.

Hypobromite.		Folin and Youngburg.		Kjeldahl.	Theoretical.
mg.	*per cent of theoretical*	*mg.*	*per cent of theoretical*	*mg.*	*mg.*
6 per cent bromine.					
1.000	93.98	1.062	99.81	1.064	1.064
1.016	95.48	1.062	99.81	1.064	
1.01	94.93	1.060	99.62		
1.015	95.39	1.063	99.91		
1.020	95.86	1.063	99.91		
1.016	95.48	1.062	99.81		
0.644	96.84	0.662	99.55	0.665	0.665
0.646	97.14	0.663	99.69	0.665	
0.643	96.69	0.663	99.69		
0.640	96.25	0.663	99.69		
10 per cent bromine.					
1.00	93.98				1.064
1.02	95.86				
1.00	93.98				
1.00	93.98				
1.01	94.93				
0.5 per cent bromine.					
1.01	94.95				1.064
1.03	96.8				
1.03	96.8				
1.02	95.86				
1.03	96.8				

These figures are in accordance with the findings of Chattaway,[3] Dowell,[4] and Krogh,[5] that there is not a quantitative liberation of nitrogen when urea is acted upon by hypobromite.

[6] Dehn, W. M., *J. Am. Chem. Soc.*, 1907, xxix, 1317.

NOTE ON THE GASOMETRIC DETERMINATION
OF UREA.

By RAYMOND L. STEHLE.

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This note is occasioned by the preceding communication,[1] for an early knowledge of the contents of which the writer is indebted to the author.

Additional analyses have been conducted on pure urea and urea oxalate solutions with the following results:

Urea oxalate N.		Urea N.	
Found.	Calculated.	Found.	Calculated.
mg.	*mg.*	*mg.*	*mg.*
38.9	38.7	193	195
39.5	38.7	198	195
74.4	74.6	198	195
75.0	74.6	193	196
73.9	74.6	193	196
73.5	74.6	193	196
38.3	38.0		

In calculating the results of urea determinations by the gasometric method described by the writer[2] a certain amount of error is involved. The solution left in the apparatus at the end of a determination is a rather concentrated one and hence it is not correct to assume that its vapor tension is the same as that of water. This erroneous assumption was made in the original description of the method because vapor tension data for hypobromite solutions were unknown to the author at the time, and the error introduced is not large enough to invalidate the method for

[1] Menaul, P., *J. Biol. Chem.*, 1922, li, 87.

[2] Stehle, R. L., *J. Biol. Chem.*, 1921, xlvii, 13.

most purposes. Recently, some data on this point in a paper by Dehn[3] have come to the writer's attention. In order to make them applicable to the method it is necessary to prepare the hypobromite solution as described by Dehn with the omission of the final step; *viz.*, doubling the volume with water. Then by using 1 cc. of the solution to be analyzed, 1 cc. of rinse water, and 2 cc. of the hypobromite solution the vapor tension of the solution in the apparatus should approximate closely the tension called for by Dehn's table. Dehn's solution is prepared by dissolving 100 gm. of NaOH in 250 cc. of water, adding 10 cc. of Br to each 100 cc., and doubling the volume with water. The vapor tensions are as follows:

Temperature.	NaOBr	H₂O	Difference.
°C.	mm.	mm.	mm.
4	1.1	6.1	5.0
8	1.8	8.0	6.2
12	3.3	10.5	7.3
16	5.3	13.6	8.3
20	8.6	17.4	8.8
24	12.8	22.2	9.4
28	18.0	28.1	10.1
32	24.0	35.4	11.4
36	29.5	44.2	14.7
40	36.0	55.0	19.0

These data were utilized in the calculation of the results reported above. The dissolved air contained in this hypobromite solution is negligible.

Concerning the work of Chattaway,[4] Dowell,[5] and Krogh,[6] all of which Menaul states to show the urea-hypobromite reaction not to be stoichiometric, the following comments are apropos. In the paper by Chattaway an explanation is suggested for the failure of the urea-hypobromite reaction to give quantitative results. When urea and hypochlorous acid react in acid solution dichloro urea is formed. This substance hydrolyses easily according to the equation

[3] Dehn, W. M., *J. Am. Chem. Soc.*, 1907, xxix, 1317.
[4] Chattaway, F. D., *Chem. News*, 1908, xcviii, 285.
[5] Dowell, C. T., *J. Am. Chem. Soc.*, 1919, xli, 124.
[6] Krogh, M., *Z. physiol. Chem.*, 1913, lxxxiv, 379.

$$\begin{matrix} & \nearrow NHCl & & & \nearrow OH & \\ CO & & + 2H_2O = & CO & & + 2NH_2Cl \\ & \searrow NHCl & & & \searrow OH & \end{matrix}$$
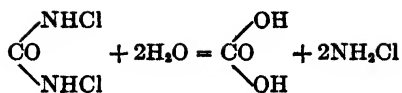
The NH_2Cl is then assumed to decompose as follows:

$$3NH_2Cl = 2NH_3 + NCl_3$$

The end-products then

" . . . . react, forming nitrogen and hydrogen chloride, the latter at once combining with the free ammonia and allowing the remaining nitrogen trichloride to escape, as this does not react with ammonium chloride. . . . In presence of alkalies, on the other hand, the reaction between the ammonia and the nitrogen trichloride goes on to completion, since the hydrochloric acid formed in it is at once fixed; no nitrogen trichloride therefore is set free, since twice as much ammonia is formed as is required to decompose it."

In order to account for the incompleteness of the urea-hypobromite reaction Chattaway continues:

"The urea, is, without doubt, converted into dichloro or dibromo urea, which is at once hydrolyzed in the manner above described. In presence of the excess of hypochlorite or hypobromite, the mono substituted ammonia formed in the hydrolysis may be further substituted to a greater or less extent, nitrogen being evolved quantitatively only when this takes place under such conditions that the amount of hydrogen attached to nitrogen in the reacting system is always sufficient to react completely with the chlorine attached to nitrogen."
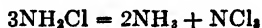
Two facts concerning Chattaway's paper are these: (1) It is not stated that the reaction necessarily falls short of being quantitative; and (2) no data are presented to show that with alkaline hypobromite nitrogen tribromide is formed. Conditions which might favor its formation are suggested but that is all. Indeed from the foregoing quotations one would be inclined to believe that the reaction would be quantitative rather than otherwise.

The paper by Dowell concerns the action of chlorine upon urea. This is somewhat different than the action of a strongly alkaline hypobromite solution and hardly justifies the conclusion that the low results of the urea-hypobromite reaction as observed in previous methods are due to the formation of nitrogen tribromide. It is worth noting that Dowell found no evidence of the formation of NH_2Cl in the hydrolysis of dichloro urea as assumed by

Chattaway.   Neither did he observe the oxidation of nitrogen to nitric or nitrous acid.   This latter fact is interesting in connection with the following comment on the work of Krogh:

Krogh[6] has studied the urea-hypobromite reaction as it is ordinarily carried out and found that when the hypobromite solution is poor in Br some CO (0.62 per cent is the highest value recorded) may be formed but that when the solution is rich in Br little or no CO is evolved and the evolution of N is also less quantitative (13.8 per cent is the greatest deficiency recorded). The low N Krogh believed to be due to the formation of oxides of N.   She obtained a qualitative test for nitric acid.   In order to learn whether similar results could be obtained in the vacuum apparatus a hypobromite solution similar to that employed by Krogh in obtaining the last mentioned result was employed.   The liberation of nitrogen was complete.   Indeed the hypobromite solution of Dehn contains more Br than that employed by Krogh yet gives satisfactory results.   It appears, therefore, that when conducted *in vacuo* the reaction does not follow the same course as when conducted at atmospheric pressure.   If carrying the reaction out *in vacuo* has no effect on the result one may wonder why Menaul's results are surprisingly independent of the hypobromite concentration instead of in accord with the results of Krogh on this point.

THE CARBOHYDRATE CONTENT OF THE SEED OF ASPARAGUS OFFICINALIS L.

By W. E. CAKE AND H. H. BARTLETT.

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INTRODUCTION.

The reserve hemicelluloses of seeds have been investigated especially by Schulze[1] and his associates, to whose numerous papers we owe most of our present knowledge of these compounds. They occur as stored foods in seeds of plants belonging to the most distantly related groups, and offer a large field for biochemical study. It has been found that in most cases the reserve hemicelluloses yield galactose and mannose upon hydrolysis, and are, therefore, either mixtures of mannan and galactan or else galactomannans. Baker and Pope[2] found that vegetable ivory, the hemicellulose from seeds of *Phytelephas*, yielded mannose and about 5 per cent as much fructose, pointing to the existence of a fructomannan. Our results, showing a large percentage of fructose among the cleavage products of asparagus seed, suggest that fructose may have been overlooked in much previous work. If this surmise should not be true, the presence or absence of fructose will afford an important criterion in the further study and classification of the hemicelluloses. In general, it may be said that little is known about the constitution of individual hemicelluloses, since methods have not been devised for differentiating them as chemical individuals. In the absence of such methods, complete analytical data, accounting for all cleavage products, may throw light upon their constitution. It is hoped

[1] Schulze, E., and associates. For list of references see Czapek, F., Biochemie der Pflanzen, Jena, 2nd edition, 1913, i, 420, 655.
[2] Baker, J. L., and Pope, T. H., *Proc. Chem. Soc.*, 1900, xvi, 72.

that the results presented in this paper will be useful along this line.

After the removal of the large oil content of asparagus seed, the hemicellulose may be extracted by dilute alkali and precipi-, tated either by acidifying or by adding alcohol. The amorphous precipitate may be somewhat purified by redissolving and re-precipitating. After drying, t turns to a dark blue color with strong iodine solution, and may quickly be washed colorless by suspending it in water and decanting two or three times.

In the analysis of asparagus seed by acid hydrolysis the polysaccharide is accounted for as mannose, fructose, glucose, and galactose. The total mannose is only 24.2 per cent, and the other three sugars total 21.9 per cent, suggesting that there is an equivalent of mannose for each equivalent of every other sugar. Since starch was not present in the seeds, nor inulin or any similar compound, and since cellulose was not attacked in the method of hydrolysis used, it is probable that the individual polysaccharide or polysaccharides of the asparagus seed are mixed, each being a condensation product of two or more hexoses. Unless the hemicellulose molecule be excessively large, it is fair to assume that a very small galactose content is derived from a polysaccharide quite distinct from that yielding the bulk of the mannose. This polysaccharide might be a galactomannan, a glucogalactan, or a simple galactan—most likely a galactomannan. The bulk of the polysaccharide might conceivably be of a single kind, a glucofructomannan, without requiring the hypothesis of a very large molecule, or it might be a mixture of two or more simpler kinds, as for example a glucomannan with a fructomannan. At any rate, the occurrence of mannose in a practically 1:1 ratio with the total remaining hexoses is distinctly suggestive of the possible manner in which the hemicellulose is constituted.

Two former papers touching on the chemistry of asparagus seed have been published—those of Reiss[3] and of Peters.[4] The latter worker estimated all the hydrolytic sugar of the seed as mannose, failing to detect the other hexoses. He did not report even galactose, as it would be inferred he did from the reference to

[3] Reiss, R., *Ber. chem. Ges.*, 1889, xxii, pt. 1, 609.

[4] Peters, W., *Arch. Pharm.*, 1902, ccxl, 53.

his paper in Czapek.[5]   The earlier paper of Reiss was the first significant investigation in the field of hemicellulose chemistry, and dealt with asparagus seed only incidentally, as one of the sources of his newly discovered sugar, "seminose," afterwards found to be the same as mannose.

Material.

The seeds of *Asparagus officinalis* L. used in this work were obtained from the firm of D. M. Ferry and Co. of Detroit, Michigan, and were of the variety "Palmetto." They were first separated from most foreign matter by picking them over, and then ground in a large grinding mill until they were fine enough to pass through a 40 mesh sieve. The sample was thoroughly mixed and kept in a sealed jar until needed for use.

Preliminary Examination.

An analysis was made of the sample so prepared according to the methods of the Association of Official Agricultural Chemists[6]

TABLE I.

Preliminary Analysis of Asparagus Seed, var. "Palmetto."

	per cent
Moisture	10.41
Ash	2.02
Ether extract	13.59
Crude protein (nitrogen × 6.25)	17.38
Crude fiber	4.94
Nitrogen-free extract (by difference)	51.66

except that for the determination of nitrogen, used in computing the "crude protein," the iodometric method of Willard and Cake[7] was used.   These analyses gave the results shown in Table I.

Part of the total nitrogen of the asparagus seeds is contained in the black seed coats.   The black pigment in these seed coats seems to be of a melanoid nature, and is being investigated further.

The relatively high percentage of ether extract, a fixed oil, is interesting.   Work has been about completed on a study of the

[5] Czapek, F., Biochemie der Pflanzen, Jena, 2nd edition, 1913, i, 421.

[6] Official and tentative methods of analysis of the Association of Official Agricultural Chemists, 1920.

[7] Willard, H. H., and Cake, W. E., *J. Am. Chem. Soc.*, 1920, xlii, 2646.

physical and chemical properties of this oil, and these results, in some respects at variance with those of Peters,[4] will be published shortly.   .

Qualitative Study of the Carbohydrate Content.

Qualitative experiments were first conducted in order to determine what sugars were produced by acid and enzyme hydrolyses of the asparagus seeds. For this purpose one portion of the seeds was boiled with 2 per cent sulfuric acid, and after neutralization with calcium carbonate and purification with lead acetate, the osazones of the sugars were prepared by heating with an excess of phenylhydrazine in dilute acetic acid solution. To prepare the sugars resulting from enzyme hydrolysis another portion of the seeds was placed in water to which a little chloroform had been added, and allowed to undergo autolysis. A portion of this solution after purification with lead acetate was heated with an excess of phenylhydrazine in dilute acetic acid solution. In both cases the osazones were separated by recrystallization into ten different fractions, and each fraction proved to contain nothing but phenylglucosazone (M. P. 208–213°). This indicated that no sugars could have been present in the original solution other than glucose, mannose, and fructose, except perhaps in mere traces.

For further identification of the sugars present their hydrazones were prepared by adding phenylhydrazine acetate to the cool neutral solutions. There separated at once the white insoluble precipitate of mannose phenylhydrazone, which after recrystallization from 60 per cent alcohol, melted at 194–195°. This hydrazone was further identified by heating with an excess of phenylhydrazine in dilute acetic acid solution, when phenylglucosazone was formed (M. P. 210°). A portion of the purified mannose phenylhydrazone was reconverted into mannose by the formaldehyde method of Ruff and Ollendorff,[8] as modified by Browne and Tollens.[9] This mannose was crystallized from 80 per cent alcohol, and the anhydrous crystals so obtained melted at 131.°

All attempts to bring any other phenylhydrazones to crystallization in the filtrate from the mannose phenylhydrazone proved

[8] Ruff, O., and Ollendorff, G., *Ber. chem. Ges.*, 1899, xxxii, 3234.
[9] Browne, C. A., Jr., and Tollens, B., *Ber. chem. Ges.*, 1902, xxxv, 1457.

futile. This filtrate, however, on heating with an excess of phenylhydrazine in dilute acetic acid solution yielded large quantities of glucose phenylosazone (M. P. 206–207°). Since the mannose had been almost quantitatively removed as the insoluble phenylhydrazone, the remaining sugar could have been only glucose or fructose or both.

A filtrate from the precipitation of mannose phenylhydrazone, containing about 25 gm. of the sugars in the form of their phenyl-hydrazones, in 200 cc. of water and 280 cc. of alcohol, was refluxed for 5 hours with 32 gm. of benzaldehyde according to the method of Herzfeld[10] and de Witt, for converting the hydrazones back into sugars. After the removal of the benzaldehydrazone, excess benzaldehyde, benzoic acid, and alcohol, the solution was boiled with a little animal charcoal and filtered. In this solution the presence of glucose was established by oxidation with 25 per cent nitric acid according to the method of Gans and Tollens.[11] On neutralization with potassium carbonate and making slightly acid with acetic acid, the characteristic potassium salt of saccharic acid crystallized out. The presence of glucose was further indicated by the optical rotation of the solutions, as shown in Table IV.

The presence of fructose was established both in the original solution resulting from the acid hydrolysis of the asparagus seeds, and also in the solution after removal of the mannose, by the Seliwanoff[12] test, of heating a few drops of each with concentrated hydrochloric acid and a little resorcinol—a bright cherry-red color developing almost immediately. The diphenyl-amine reaction of Ihl-Pechmann carried out as directed by Jolles[13] also gave positive results—a deep blue color being developed in 2 minutes. In addition, the methyl fructosazone was prepared from the solution after the removal of mannose, according to the directions of Neuberg.[14] This osazone melted at 158–159°.

[10] Herzfeld, A., *Ber. chem. Ges.*, 1895, xxviii, 442.

[11] Gans, R., and Tollens, B., *Ann. Chem.*, 1888, ccxlix, 219.

[12] Seliwanoff, T., *Ber. chem. Ges.*, 1887, xx, 181.

[13] Jolles, A., *Ber. pharm. Ges.*, 1909–10, xix, 484.

[14] Neuberg, C., *Ber. chem. Ges.*, 1902, xxxv, 959.

Quantitative Examination of the Carbohydrates.

The constituents of the carbohydrate content of asparagus seeds were quantitatively determined in the ether-extracted samples according to the following methods:

Total Reducing Sugars on Acid Hydrolysis.—The sample was re-fluxed for $2\frac{1}{2}$ hours with 3 per cent hydrochloric acid. After neutralization the reducing sugars were determined in an aliquot part by means of Fehling's solution. The precipitation was carried out according to the directions of Munson and Walker,[15] and the copper in this precipitate estimated by the iodometric method of Low.[16]

Reducing Sugars Resultant from Hydrolysis of Polysaccharides.—The oil-free sample was extracted with 150 cc. of 10 per cent alcohol on a hardened filter paper, the insoluble residue hydrolyzed, and the reducing sugars in the solution so obtained were estimated by Fehling's solution.

Free Sugars.—The difference between the total reducing sugars on acid hydrolysis and the reducing sugars resulting from the acid hydrolysis of insoluble polysaccharides gave the free sugar content. (It became obvious from the analytical results that none of the free sugar was mannose.)

Starch.—The oil-free seeds gave no blue color on treatment with iodine, nor did malt diastase affect the ether-extracted sample.

Pentosans.—The sample was boiled with 12 per cent hydrochloric acid and the furfuraldehyde in the distillate estimated as the phloroglucide according to the directions given in the Official Methods.[6] The pentosan results are apt to be a little high since the hexoses on distillation with hydrochloric acid yield small amounts of furfuraldehyde, a fact of especial importance in connection with this work, since mannose is especially apt to give rise to furfuraldehyde. Fischer and Hirschberger[17] have shown that this change occurs even when a 5 per cent solution of mannose is heated in a sealed tube at 140° for 4 hours.

[15] Munson, L. S., and Walker, P. H., *J. Am. Chem. Soc.*, 1906, xxviii, 663. Walker, P. H., *J. Am. Chem. Soc.*, 1907, xxix, 541.

[16] Low, A. H., *J. Am. Chem. Soc.*, 1902, xxiv, 1082.

[17] Fischer, E., and Hirschberger, J., *Ber. chem. Ges.*, 1889, xxii, 365.

Galactans.—The sample was treated with 25 per cent nitric acid and the mucic acid estimated by the method given in the Official Methods.[6]

Mannose.—The oil-free sample was refluxed for $2\frac{1}{2}$ hours with 3 per cent hydrochloric acid. After cooling, the solution was neutralized with dilute sodium hydroxide, then 2 cc. acetic acid were added. The solution was next filtered and the residue thoroughly washed with water. The filtrate was concentrated on a water bath to about 30 cc. and then cooled   The mannose

TABLE II.

Determination of the Total Free Sugar and of the Several Classes of Polysaccharides.

	Non-summable percentages.	Summable percentages.
	per cent	*per cent*
Total sugars on acid hydrolysis..................	54.5	
Sugars from hydrolysis of polysaccharides......	50.4	
.Free sugars....................................		4.1
Condensed sugars..............................	45.4	
Pentosans.....................................		2.0
Galactans.....................................		0.4
Polysaccharides yielding glucose, fructose, and mannose....................................		43.0
Total carbohydrates.....................		49.5

in this solution was now determined as mannose phenylhydrazone according to the method of Bourquelot and Hérissey.[18]

Glucose: Fructose Ratio.—The oil-free sample was hydrolyzed with 3 per cent hydrochloric acid. After neutralization with dilute sodium hydroxide, and making slightly acid with acetic acid, the solution was purified with lead acetate. The solution after filtration was freed from excess lead by hydrogen sulfide. After filtration the hydrogen sulfide was removed by blowing a stream of air through the solution, which was finally heated to boiling. The total sugars were determined in an aliquot portion. The aldoses were determined in another aliquot by the hypoiodite

[18] Bourquelot, E., and Hérissey, H., *Compt. rend. Acad.*, 1899, cxxix, 339.

method of Willstätter and Schudel.[19]   Since the ratio of mannose
to total sugars had already been determined, it was simply a matter

TABLE III.

Determination of Aldoses and Ketoses by Willstätter's Hypoiodite Method.

	Non-summable percentages.	Summable percentages.
	per cent	*per cent*
Aldoses in total sugars.........	46.8	
" " insoluble polysaccharides...........	43.8	
Free aldoses...................................		3.0
Condensed aldoses.................		39.4
Ketoses in total sugars.......................	8.0	
" " condensed sugars	7.0	
Free ketoses..................................		1.0
Condensed ketoses............................		6.3
Total..		49.7

TABLE IV.

Determination of Glucose, Fructose, and Mannose from the Optical Activity.

	Non-summable percentages.	Summable percentages.
	per cent	*per cent*
Mannose in total sugars........................	24.2	
" " polysaccharides.....................	24.2	
Free mannose...................................		0.0
Condensed mannose............................		21.8
Glucose in total sugars.........................	20.1	
" " polysaccharides.....................	16.8	
Free glucose...................................		3.3
Condensed glucose.............................		15.1
Fructose in total sugars........................	8.5	
" " polysaccharides.....................	7.1	
Free fructose..................................		1.4
Condensed fructose		6.4
Total..		48.0

of computation to determine the glucose: fructose: mannose
ratios.   The same process was carried through after the removal
of the free sugars.

[19] Willstätter, R., and Schudel, G., *Ber. Chem. Ges.*, 1918, li, 782.

Knowing the total sugar content and the mannose content of a solution of glucose, fructose, and mannose, it is possible to calculate the ratio of these three sugars from the optical activity of their solution. Accordingly, determinations were made of the optical activity of a purified solution of the total sugars obtained by acid hydrolysis. The same was done with the solution resulting from acid hydrolysis of the sample after the removal of the free sugars.

RESULTS.

In the determination of the glucose: fructose ratio, 2.24 per cent was subtracted from the sugar content of both solutions

TABLE V.

General Summary of Analysis of Asparagus Seed, var. "Palmetto."

	per cent
Moisture	10.41
Ash	2.02
Oils	13.59
Crude protein	17.38
Crude fiber	4.94
Pentosans	2.02
Galactans	0.42
Starch	0.00
Mannose	0.00
Glucose	3.3
Fructose	1.4
Condensed mannose	21.8
" glucose	15.1
" fructose	6.4
Total	98.8

to make allowance for the pentoses present. Furthermore, it was assumed that the effect of this small pentose content on the plane of polarization was zero. In event that all the furfuraldehyde in the pentosan determination resulted from the mannose present, the glucose and fructose estimations are low—about 95 per cent of what they should be.

SUMMARY.

1. A quantitative examination of asparagus seed (*Asparagus officinalis* L., var. "Palmetto") has shown that the reserve carbohydrate is in the form of hemicelluloses, which give, on hydrolysis, mannose, glucose, fructose, and galactose.

2. The galactose is in such small quantity that it appears likely that it forms part of a different hemicellulose from that which constitutes the bulk of the reserve carbohydrate.

3. The mannose is in a ratio of 1:1 to the total remaining hexoses. The absence in appreciable quantity of carbohydrates having the properties of cellulose, starch, and inulin makes it seem likely that the hemicelluloses are either glucomannans occurring with fructomannans or else glucofructomannans.

4. The proof that fructose is one of the cleavage products derived from the hemicellulose of asparagus seed confirms the single previous report of fructose among the hydrolytic products of a hemicellulose, namely, the report by Baker and Pope[2] in the case of *Phytelephas*.

A CHEMICAL STUDY OF THE PROTEINS OF THE ADSUKI BEAN, PHASEOLUS ANGULARIS.*

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Chemical studies of the proteins of various beans, including the navy,[1] lima,[1] mung (1), Chinese velvet (2), Georgia velvet (3), and the jack beans (4), have shown that nearly all of them contain two globulins, one occurring in relatively small amounts and the other constituting the chief protein of the seeds. The former have been designated α-globulins and the latter β-globulins. The α-globulins were separated by addition of solid ammonium sulfate to the sodium chloride extracts of the meal in an amount sufficient to make the solution from 0.3 to 0.4 saturated. Small intermediate fractions consisting of a mixture of the two globulins were then removed by further addition of ammonium sulfate to the filtrates from the α-globulins until the solutions were about 0.6 saturated. These intermediate fractions were discarded, and from their filtrates the β-globulins were precipitated by complete saturation with ammonium sulfate.

The α-globulins differ chiefly from the β-globulins in having less nitrogen and a much higher sulfur content. The corresponding globulins obtained from the various beans which have been

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* A preliminary report of this paper was presented at the sixty-second meeting of the American Chemical Society held in New York City, September 6 to 10, 1921 (*cf. Science*, 1921, liv, 444).

The adsuki beans used in the experiments described in this paper were furnished by the Bureau of Plant Industry, Department of Agriculture, and were of the maroon variety. A complete description of the adsuki bean is given by Piper and Morse (Piper, C. V., and Morse, W. J., *U. S. Dept. Agric., Bull. 119,* 1914).

[1] Chemical studies of the navy and lima beans are still in progress, and the results will be published later.

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thus far examined, especially those of the *Phaseolus* genus, bear a close resemblance to one another in both their general properties and chemical composition.   Data other than chemical behavior and composition, however, will be necessary to show whether or not they are identical.   Work along this line is now in progress.

A similarity in the proteins of these beans has also been shown by a study of their nutritive values as found by feeding experiments with albino rats.   The proteins of the navy (5) and lima (6) beans were found to lack two factors necessary to make them available for the normal nutrition of the rats: first, a deficiency of cystine, which was corrected by the addition of 0.3 per cent of this amino-acid to a diet adequate in other respects; second, cooking—without which growth could not be obtained.   In the case of the adsuki bean (7), however, it was found that while its proteins were deficient in cystine, cooking was not required. This difference in the nutritive behavior of the adsuki bean makes a further chemical study of its proteins of additional interest.

The adsuki bean is one of five oriental species of beans which at various times have been introduced into the United States. Its cultivation was originally confined to Japan, Manchuria, China, and Korea.   Next to the soy bean it seems to be the most important legume grown in Japan, and commands a higher price than any other bean.   At the Arlington farms of the United States Department of Agriculture, in experimental tests, average yields of about 22 bushels per acre were obtained.

The beans used for the experiments described in this paper were identified as *Phaseolus angularis*.   Piper and Morse (8) have called attention to the fact that

"While the species is clearly and abundantly distinct, it has been confused with related species by most botanists. . . . .   In most Japanese botanical works the adsuki bean is confused with the mung, and therefore called *Phaseolus mungo* or *Phaseolus radiatus*, from both of which it differs greatly."

The proteins of the Japanese adsuki bean were first studied in 1897 by Osborne and Campbell (9), who isolated, by dialysis of a 10 per cent sodium chloride extract of the bean, a globulin the properties and composition of which closely resembled those of

phaseolin, the chief globulin of the navy bean. Later Osborne and Harris (10) made a nitrogen distribution in several preparations of both this globulin and phaseolin, and found that the preparations of the adsuki bean globulin gave uniformly higher results for the basic nitrogen. They also isolated a small amount of a substance from the extract after all of the globulin had been removed by dialysis in water, by further dialysis against alcohol. This protein resembled in its composition the legumelins obtained from the pea, vetch, lentil, and other leguminous seeds.

The adsuki bean meal used for the preparation of the proteins described in this paper contained 21.13 per cent of protein (N × 6.25). Preliminary experiments showed that the maximum amount of protein was extracted by thoroughly mixing the meal with aqueous 5 per cent sodium chloride solution in the proportion of 4 cc. of solvent to each gram of meal, and allowing the mixture to stand for 40 hours in cold storage at 1–3°C. It was found that if the extraction was allowed to take place at room temperature, a smaller yield of the α-globulin was obtained. This globulin denatures readily, changing into an insoluble form, a tendency which is less marked at a low temperature. In this way 79 per cent of the total protein in the meal was dissolved, or 16.7 per cent, based on the amount of meal used.

By applying to the adsuki bean the same methods which have been used in this laboratory to separate the globulins of the mung, navy, and lima beans, we have again been able to isolate two globulins closely resembling the globulins obtained from those beans. Both globulins gave positive tests for tryptophane, and 2.13 per cent of tyrosine was isolated from the β-compound. The difference in composition of these two globulins is shown by the following percentages (Table I) which are the averages of those for several preparations of each protein.

Determination of the diamino-acids by the Van Slyke method showed that the β-globulin contained more arginine, and about half as much cystine as the α-globulin, while the percentages of histidine and lysine found were practically the same. Arginine, histidine, and lysine were also determined in the β-globulin by the direct method of Kossel and Patten (11). The values obtained by the Van Slyke method were in all cases higher than those

obtained by the direct method, especially that for lysine.   The percentages of the diamino-acids are summarized in Table II.

After removal of all of the globulins from a distilled water extract of the bean meal, a small amount of an albumin was obtained by heating the dialysate for 2 hours at 70°C.

<div align="center">

TABLE I.

Average Composition of the α- and β-Globulins.

</div>

	α-Globulin.	β-Globulin.
	per cent	*per cent*
C..	52.75	53.57
H..	6.97	6.79
N..	15.64	16.46
S..	1.21	0.40

<div align="center">

TABLE II.

Percentages of the Diamino-Acids in the α- and β-Globulins.

</div>

Amino-acid.	α-Globulin.	β-Globulin.	
	Van Slyke method.	Van Slyke method.	Direct method of Kossel and Patten.
	per cent	*per cent*	*per cent*
Arginine...........................	5.45	7.00	5.30
Histidine..........................	2.25	2.51	1.76
Lysine.............................	8.30	8.41	4.18
Cystine...........................	1.63	0.86	

<div align="center">

EXPERIMENTAL.

</div>

Preliminary Experiments.—The beans were ground to a fine meal in a power driven pulverizing mill.   Extractions were then made, using small samples of the meal and varying concentrations of sodium chloride in distilled water in the proportion of 4 cc. of solvent to each gram of meal.   The mixtures were allowed to stand at a low temperature for 40 hours, and nitrogen[2] was

determined in aliquot portions of the extracts. The results (Table III) show that an extraction with 5 per cent sodium chloride solution for a period of 40 hours gave the maximum yield of protein.

Precipitation experiments made with small quantities of the clear sodium chloride extract of the meal showed that some of the protein was precipitated at 0.3 of saturation with ammonium sulfate (Preparation I). Other fractions were obtained at 0.45 (Preparation II), and 0.65 of saturation (Preparation III), and a final fraction at 0.7 to complete saturation (Preparation IV).

TABLE III.

Extraction Experiments. *

Solvent.	Protein extracted from meal (N × 6.25).
	per cent
Distilled water..............................	7.25
0.5 per cent NaCl.............................	11.88
1.0 " " "	13.81
1.5 " " "	14.44
2.0 " " "	14.69
2.5 " " "	15.31
3.0 " " "	15.69
3.5 " " "	16.56
4.0 " " "	16.63
5.0 " " "	16.75
7.0 " " "	16.06
10.0 " " "	15.25

* The extractions were carried out at 1–3°C. for a period of 40 hours each.

These various fractions were filtered and well washed with solutions of the same salts and concentrations from which they had been precipitated. They were then redissolved, dialyzed, washed, and dried in the usual way. These preparations contained 1.28, 0.95, 0.90, and 0.43 per cent of sulfur, respectively. These results show that two globulins, represented by Preparations I and IV, were present, differing markedly in their sulfur content, while Preparations II and III consisted of mixtures of the two globulins.

Preparation of the α-Globulin.—Extractions were made, using from 1 to 9 kilos of meal and 5 per cent sodium chloride solution.

The meal was thoroughly mixed with the solvent, and the mixtures allowed to stand for about 40 hours in a cold storage room at 1-3°C. Dry filter paper scraps were then pulped in the mixtures until a consistency suitable for pressing was obtained. After pressing in muslin bags, the expressed liquors were filtered through a mat of paper pulp on a Buchner funnel. The clear filtrates were then made 0.3 saturated by addition of solid ammonium sulfate and the precipitate was allowed to settle by standing for 24 hours. The precipitates were filtered on folded filter papers and washed with large volumes of 5 per cent sodium chloride solution which had been previously made 0.3 saturated with ammonium sulfate. The precipitates thus obtained were found

TABLE IV.

Averages of Duplicate Analyses of the α-Globulin.

	Preparation.					
	I	II	III	IV	V	Average.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
C....................	52.99	52.69	52.62	52.75	52.71	52.75
H....................	7.17	6.87	7.00	6.85	6.97	6.97
N....................	15.75	15.69	15.50	15.60	15.64	15.64
S....................	1.28	1.29	1.12	1.23	1.13	1.21
O....................	22.81	23.46	23.76	23.57	23.55	23.43
Moisture.............	8.69	5.99	5.83	5.29	6.35	
Ash.................	0.77	0.42	0.54	0.37	0.47	

to denature rapidly, so that considerable portions would not redissolve in 5 per cent sodium chloride solution. The dissolved portions were dialyzed against chilled running water for 9 days. In the case of Preparations I and V (Table IV), the denatured portions were mixed with the soluble portion and dialyzed together. After dialysis the preparations were washed, and dried with alcohol and ether in the usual way. An average yield of 0.35 per cent of the meal used was obtained. Averages of duplicate analyses of the five preparations of the α-globulin are given in Table IV. Moisture and ash were determined in all of the preparations, and the analyses calculated on the ash- and moisture-free basis.

Preparation of β-Globulin.—The filtrates from the original precipitates of the α-globulin, which were 0.3 saturated with ammonium sulfate, were measured and made 0.65 saturated. The small amounts of precipitates were filtered off and discarded, as preliminary experiments had shown that these fractions consisted of a mixture of the α- and β-globulins. From the filtrates of the intermediate fractions the β-globulin was precipitated by adding ammonium sulfate to saturation. The purification and drying of this material were carried out as described for the α-globulin. The average yield was 2.75 per cent of the meal extracted. The elementary composition of seven preparations is given in Table V.

TABLE V.

Averages of Duplicate Analyses of the β-Globulin.

	Preparation.							
	I	II	III	IV	V	VI	VII	Average.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
C....................	54.17	53.91	53.05	53.62	53.24	53.35	53.62	53.57
H....................	6.64	6.94	6.89	6.92	6.62	6.74	6.77	6.79
N....................	16.38	16.62	16.45	16.57	16.37	16.44	16.37	16.46
S....................	0.46	0.41	0.36	0.35	0.41	0.41	0.37	0.40
O....................	22.36	22.12	23.25	22.54	23.36	23.06	22.87	22.78
Moisture...........	6.76	5.31	5.39	4.66	5.24	5.67	5.24	
Ash................	0.43	0.36	0.32	0.15	0.26	0.21	0.21	

Properties of the Globulins.—The finished preparations are light, dusty powders, the α-globulin ranging from a pale gray to a dark gray color, while the other is of a very light gray to an ivory white. Apparently the former, being the first to precipitate from the meal extract carries with it some of the coloring matter derived from the hulls of the beans. The α-globulin was found to coagulate at about 88°C. It gave a faintly positive test for tryptophane by the Hopkins and Cole reagent, and a positive test for tyrosine with Millon's reagent. The β-globulin coagulated on heating for 10 minutes at 97°C., and gave a strongly positive test for tryptophane. The precipitation limits with ammonium sulfate have already been noted.

Analyses of the α- and β-Globulins by the Van Slyke Method.—
Duplicate samples of about 3 gm. of each of the globulins were
hydrolyzed by boiling for about 30 hours with 150 cc. of 20 per

TABLE VI.

*Distribution of Nitrogen in the α-Globulin as Determined by the Van Slyke
Method.**

Sample I, ash- and moisture-free, 2.8343 gm. protein, 0.4422 gm. nitrogen.†
Sample II, "   "   "   "   2.8302 "   "   0.4415 "   "

	Preparation.				
	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N....................	0.0444	0.0443	10.05	10.03	10.04
Humin N adsorbed by lime...	0.0061	0.0063	1.38	1.43	1.40
Humin N in ether-amyl alcohol extract................	0.0005	0.0006	0.11	0.13	0.12
Cystine N....................	0.0055	0.0053	1.23	1.19	1.21
Arginine N..................	0.0499	0.0495	11.28	11.22	11.25
Histidine N.................	0.0170	0.0177	3.83	4.01	3.92
Lysine N....................	0.0451	0.0450	10.20	10.19	10.20
Amino N of filtrate..........	0.2585	0.2596	58.46	58.80	58.63
Non-amino N of filtrate......	0.0167	0.0156	3.78	3.53	3.66
Total N regained..........	0.4437	0.4439	100.32	100.53	100.43

* Nitrogen figures corrected for the solubility of the bases.
† Nitrogen content of protein, 15.60 per cent.

TABLE VII.

Basic Amino-Acids in the α-Globulin.

Amino-acid.	I	II	Average.
	per cent	per cent	per cent
Arginine...........................	5.47	5.44	5.45
Histidine..........................	2.21	2.31	2.25
Lysine.............................	8.30	8.29	8.30
Cystine............................	1.66	1.61	1.63

cent hydrochloric acid. The phosphotungstates of the bases
were decomposed by the amyl alcohol-ether method (12). The
results of the analyses are given in Tables VI, VII, VIII, IX,
and X.

TABLE VIII.

Distribution of Nitrogen in the β-Globulin as Determined by the Van Slyke Method. *

Sample I, ash- and moisture-free, 2.8557 gm. protein, 0.4732 gm. nitrogen.†
Sample II, "   "   "   "   2.8557 "   "   0.4732 "   . "

	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N....................	0.0517	0.0515	10.92	10.89	10.91
Humin N adsorbed by lime...	0.0039	0.0041	0.83	0.86	0.84
Humin N in ether-amyl alcohol extract................	0.0001	0.0004	0.02	0.07	0.05
Cystine N...................	0.0029	0.0028	0.61	0.59	0.60
Arginine N..................	0.0653	0.0635	13.80	13.42	13.61
Histidine N.................	0.0183	0.0204	3.87	4.31	4.09
Lysine N....................	0.0458	0.0460	9.66	9.85	9.75
Amino N of filtrate..........	0.2643	0.2589	55.85	54.71	55.28
Non-amino N of filtrate.......	0.0170	0.0208	3.60	4.40	4.00
Total N regained...........	0.4692	0.4690	99.16	99.10	99.13

* Nitrogen corrected for solubility of the bases.
† Nitrogen content, 16.57 per cent.

TABLE IX.

Basic Amino-Acids in the β-Globulin.

Amino-acid.	I	II	Average.
	per cent	per cent	per cent
Arginine..........................	7.10	6.90	7.00
Histidine.........................	2.37	2.64	2.51
Lysine............................	8.33	8.48	8.41
Cystine.........	0.87	0.84	0.86

TABLE X.

Distribution of Nitrogen in the α- and β-Globulins as Calculated from the Van Slyke Analyses in Terms of Per Cent of the Proteins.

N	α-Globulin.			β-Globulin.		
	I	II	Average.	I	II	Average.
	per cent	per cent	per cent	per cent	per cent	per cent
Amide........,..... .	1.57	1.57	1.57	1.81	1.80	1.81
Humin...............	0.23	0.24	0.24	0.16	0.15	0.15
Basic................	4.15	4.15	4.15	4.63	4.67	4.65
Non-basic............	9.71	9.72	9.71	9.75	9.79	9.77
Total.............	15.66	15.68	15.67	16.35	16.41	16.38

Determination of Tyrosine and the Basic Amino-Acids in the β-Globulin by the Direct Method of Kossel and Patten.

Tyrosine.—50 gm. of the β-globulin equivalent to 47.6 gm. of the ash- and moisture-free protein were hydrolyzed by boiling for 48 hours with a mixture of 150 gm. of concentrated sulfuric acid and 300 cc. of water. The solution, freed from sulfuric acid and concentrated until crystals began to form, yielded 1.01 gm. of tyrosine equivalent to 2.13 per cent of the protein. Without recrystallization it was analyzed with the following results:

0.1335 gm. substance required 7.60 cc. of 0.1 N acid.
$C_9H_{11}O_3N$. Calculated. N 7.73.
Found.   " 7.99.

The filtrates and washings from the tyrosine were used for determinations of the bases according to the method of Kossel and Patten.

Histidine.—The solution of the histidine = 500 cc.

50 cc. solution required 16.15 cc. 0.1 N acid = 0.2267 gm. N in 500 cc. = 0.8368 gm. histidine or 1.76 per cent in the protein.

The histidine was converted into the dihydrochloride for identification.

0.1230 gm. substance: 0.1534 gm. silver chloride.
0.0792 "   "   0.0915 " carbon dioxide and 0.0372 gm. water.
$C_6H_{11}O_2N_3Cl_2$. Calculated. Cl 31.14, C 31.58, H 4.86.
Found.   " 30.85, " 31.52, " 5.26.

Arginine.—The solution of arginine = 1,000 cc.

50 cc. solution required 27.70 cc. 0.1 N acid = 0.7778 gm. N in 1,000 cc. = 2.4140 gm. arginine + 0.1080 gm. = 2.5220 gm. or 5.30 per cent.

The arginine was converted into the copper nitrate double salt for identification.

0.2023 gm. substance: 0.0278 gm. copper oxide.
$C_{12}H_{28}O_4N_8Cu(NO_3)_2\cdot3H_2O$. Calculated. Cu 10.79.
Found.   " 10.98.

Lysine.—The lysine picrate weighed 5.10 gm. equivalent to 1.99 gm. lysine, or 4.18 per cent.

0.1638 gm. substance required 21.65 cc. 0.1 N acid.
$C_4H_{14}O_2N_2 \cdot C_6H_3O_7N_3$. Calculated. N 18.67.
                                Found. " 18.47.

A summary of the percentages of amino-acids in the two globulins is given in Table II.

The Albumin.

A small amount of albumin was also isolated from the adsuki bean. The meal was extracted for 40 hours at 1–3°C., with three times its weight of distilled water, and most of the globulins were removed from the clear, filtered extract by dialysis for 18 days. A further separation of a small amount of globulin was effected by passing into the filtered solution after dialysis a stream of carbon dioxide for 3½ hours. After filtering, the clear solution was heated for 2 hours at 70°C., and the coagulated albumin washed and dried in the usual way. The yield was about 0.05 per cent of the original meal. Owing to the small amount of material available, its analysis was limited to that of its sulfur content, which was found to be 2.19 per cent.

SUMMARY.

The adsuki bean contains about 21.13 per cent of protein (N × 6.25), 16.7 per cent of which is extracted by means of a 5 per cent aqueous sodium chloride solution.

By fractional precipitation of the sodium chloride extracts with ammonium sulfate two globulins, designated as the α- and β-globulins, have been isolated. The former was precipitated by addition of ammonium sulfate in sufficient amount to make the original extract 0.3 saturated. A small fraction consisting of a mixture of the two globulins was separated from the filtrate by increasing the concentration of ammonium sulfate up to 0.65 of saturation. This fraction was discarded, and the β-globulin precipitated by making the solution completely saturated. A small amount of an albumin was found in distilled water extracts of the bean, after the globulins had been removed.

The two globulins differ markedly in their sulfur and nitrogen content and in their nitrogen distribution as determined by the method of Van Slyke.

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CLINICAL METHOD FOR THE ESTIMATION OF CHLORIDES IN BLOOD.

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All clinicians readily appreciate the importance of sodium chloride estimation in affording very valuable data for the diagnosis, treatment, and prognosis of certain diseases and conditions. The value of a reliable and convenient clinical method is, therefore, obvious.

An ideal clinical method is one that is easy of manipulation, accurate, rapid, and one that utilizes as little apparatus and reagents as possible. The estimation of chlorides may be carried out in several ways: gravimetrically, volumetrically, and by iodimetry.

A gravimetric method is perhaps always best in that it is most exact, but for clinical purposes it is unpractical for the reason that it is time-consuming, requires more or less apparatus (balance, etc.), skill, and rather large amounts of material. Iodimetric methods are very good but irksome and call for too many reagents. The proposed method is very simple, requiring very little glassware and reagents such as an ordinary office laboratory can readily provide.

A factor generally neglected in blood chemistry is that for many determinations only the plasma should be analyzed. This was emphasized by Kuttner[1] in describing his uric acid test. It is particularly true of the chloride content. Some observations recorded in the literature give the chloride content of whole blood, others the content of plasma or serum. As early as 1850 Carl Schmidt[2] gave figures for the chloride content of both whole blood

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[1] Kuttner, T., *J. Am. Med. Assn.*, 1915, lxv, 245; 1916, lxvi, 1370.

[2] Schmidt, C., Charakteristik der Epidemischen Cholera, Leipsic and Mitau, 1850.

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and plasma and found that the chloride content as NaCl is about 0.12 per cent higher in plasma. Recent observations by Van Slyke and Cullen[3] confirmed by McLean[4] and Myers and Short[5] call attention to the change (increase) of NaCl in plasma if plasma remains in contact with the red cells. They interpret this increase as due to a loss in bicarbonate. Since, however, the plasma rather than the whole blood bathes the tissues of the body, it seems only logical to study the chloride content of plasma only; unless, however, the plasma is separated immediately there appears as stated, and from my own observations, a gradual increase in the chloride content due to a decreasing CO_2 tension.

The method to be described has been compared with the standard one of McLean and Van Slyke. The tests were performed under similar conditions and these are the results:

NaCl per 100 cc.	
McLean and Van Slyke's method.	Author's method.
mg.	*mg.*
585.0	581.1
650.8	645.4
628.8	625.1
592.3	589.3
606.9	603.5
731.2	728.1
599.6	592.3

A salt solution was also made 100 cc. = 0.509 gm. NaCl according to Folin's method. Several blanks were run using this solution with the following results:

Solution No.	NaCl per 100 cc.
	mg.
1	504.5
2	504.5
3	504.5
4	504.5

[3] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 317.
[4] McLean, F. C., *J. Exp. Med.*, 1915, xxii, 212.
[5] Myers, V. C., and Short, J. J., *J. Biol. Chem.*, 1920, xliv, 47.

The same solution was again added in 1 and 2 cc. quantities to blood, the NaCl volume of which was known,—results were obtained as follows:

	No. 1 NaCl per 100 cc.		No. 2 NaCl per 100 cc.
	mgm.		*mgm.*
Blood................	0.6435	Blood.................	0.6727
" 1 cc...........	1.1475	" 1 cc...........	1.1720
" 2 cc...........	1.6525	" 2 cc...........	1.6765

Very careful tests were conducted for the detection of interfering constituents such as sulfates, phosphates, and uric acid. These substances were not found.

Briefly, the protein of the plasma is precipitated by a neutral precipitant, made up to volume, filtered, and titrated directly against a standard silver nitrate solution. A chart is then consulted, avoiding all calculation.

Method.

Reagents.—The following reagents are used: Aluminum cream, 0.02 N silver nitrate, and 5 per cent solution potassium chromate.

Aluminum Cream.—It is best to prepare this and not to rely upon commercial products. To a liter of saturated aluminum alum (c. p. not necessary) add concentrated NH_4OH until complete precipitation. Boil till weakly alkaline to litmus. Let stand to settle. Wash by decantation till supernatant fluid is neutral to phenolphthalein. Adjust to volume of 800 cc. Place in well corked bottle. Procedure takes about 24 hours. This cream is free of chlorides.

Silver Nitrate.—This is made by accurately measuring from a burette 20 cc. of 0.10 N solution into a 100 cc. volumetric flask and adding distilled water up to the mark. Kept in a dark bottle, this solution does not deteriorate upon short standing.

Technique.

Blood must be taken on a fasting stomach. It is important to remember that the plasma must be separated immediately to obtain correct results. Pipette 1 cc. of clear plasma (slight

AgNO₃ used.	NaCl per 100 cc.	AgNO₃ used.	NaCl per 100 cc
cc.	*mg.*	*cc.*	*mg.*
2.00	292.5	4.00	585.0
2.05	299.8	4.05	592.3
2.10	307.1	4.10	599.6
2.15	314.4	4.15	606.9
2.20	321.6	4.20	614.2
2.25	329.0	4.25	621.5
2.30	336.3	4.30	628.8
2.35	343.8	4.35	636.1
2.40	351.0	4.40	643.5
2.45	358.3	4.45	650.8
2.50	365.6	4.50	658.1
2.55	372.9	4.55	665.4
2.60	380.2	4.60	672.7
2.65	387.5	4.65	680.0
2.70	394.7	4.70	687.3
2.75	402.1	4.75	694.6
2.80	409.5	4.80	702.0
2.85	416.8	4.85	709.3
2.90	424.1	4.90	716.6
2.95	431.4	4.95	723.9
3.00	438.7	5.00	731.2
3.05	445.6	5.05	738.5
3.10	453.3	5.10	745.8
3.15	460.6	5.15	753.1
3.20	468.0	5.20	760.5
3.25	475.3	5.25	767.8
3.30	482.6	5.30	775.1
3.35	488.9	5.35	782.4
3.40	497.2	5.40	789.7
3.45	504.5	5.45	797.0
3.50	511.8	5.50	804.3
3.55	519.1	5.55	811.6
3.60	526.5	5.60	819.0
3.65	533.8	5.65	826.3
3.70	541.1	5.70	833.6
3.75	548.4	5.75	840.9
3.80	555.7	5.80	848.2
3.85	563.0	5.85	855.5
3.90	570.3	5.90	862.8
3.95	577.6	5.95	870.1
		6.00	877.5

Computation; 1 cc. 20.0 N AgNO₃ ⇌ 1.170 gm. NaCl. Since the filtrate represents ⅜ of 1 cc. of the plasma it is necessary to take ¼ of the result obtained. Titration × 1.170 × ¼ × 100 = mg. NaCl per 100 cc.

hemolysis does not interfere) into a 25 cc. volumetric flask containing about 10 cc. water. Add from a graduate 3 cc. of aluminum cream and make up to volume with water. Shake well, stand 10 minutes. Filter through 5 cm. dry filter paper. 22 cc. of filtrate should be obtained and filtrate should be water-clear; slight color, however, will not interfere. Pipette 20 cc. of this filtrate into a 50 cc. beaker or Erlenmeyer flask. Add 5 drops of the 5 per cent potassium chromate. Titrate until the yellow color is changed to a first tint of dirty brown. Note number of cc. used. Consult chart for reading. For very exact work, deduct 0.05 cc. from reading for the reason that this end-point is actually silver chromate. For clinical use, this is not necessary. Milk may be done in exactly the same way.

The determination of chlorides in blood has as yet found little applicability in clinical medicine. The value of such an estimation, however, is not to be denied, particularly where diet restrictions are to be followed. Generally, such conditions as cardiac diseases, anemia, and malignancy have shown high chloride content, while fevers, diabetes, and pneumonia have shown low figures.

In conclusion, I wish to thank Dr. H. R. Miller for his interest and ever ready advice.

STUDIES ON THE AMINO-ACID NITROGEN CONTENT OF THE BLOOD.

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It has been shown by various workers, especially by Van Slyke and Meyer (1), György and Zunz (2), and Bock (3) that the amino-acid content of the blood of fasting animals for each species is fairly constant. It was further known that this constancy is maintained with remarkable tenacity except during digestion, when a transient increase occurs. Under special conditions, however, certain deviations from this constant may take place. The purpose of this paper is to study the conditions under which these deviations might develop. Experiments were carried out on animals and on the bloods of various ward cases. To control the results and to get further explanations we have studied also the contents of blood sugar, urea nitrogen, and non-protein nitrogen.

The amino-acid nitrogen was determined by the method described previously by one of us in this Journal (4).

The blood sugar content was determined by the Lewis and Benedict (5) method modified by Myers and Bailey (6). The urea nitrogen content of bloods was estimated by the Van Slyke and Cullen (7) method with slight modifications. The non-protein nitrogen was determined by Bang's micro method (8).

Physiological Amino-Acid Nitrogen Content of the Blood.

Van Slyke and Meyer, using the alcohol precipitation method, found from 3 to 5 mg. of amino-acid nitrogen per 100 cc. of blood in dogs which had been fasting from 20 to 24 hours. György and Zunz, using the same method, and removing urea by treatment with soy bean and subsequent aeration, obtained 4.8 mg. of

amino-acid nitrogen in 100 cc. of blood from dogs fasted for 24 hours. Bock, using the heat-trichloroacetic acid precipitation method, found an average of 7.47 mg. of amino-acid nitrogen per 100 cc. of dog's blood, 8.43 mg. from pigs, 8.68 mg. from cats, 7.63 mg. from sheep, 6.84 mg. from calves, 6.58 mg. from oxen, and 7.13 mg. from human venous blood. As Okada reported in a previous paper that there was no appreciable difference between the results of the heat-trichloroacetic acid method and the heat-kaolin procedure, we first tried to see whether we could get the average amino-acid nitrogen content in the blood of dogs that Bock found. We always used animals that were fasting from 20 to 48 hours and found an average of 7.3 mg. of amino-acid nitrogen per 100 cc. of blood, coinciding fairly well with the result of Bock. The content of amino-acid nitrogen of the blood of rabbits is somewhat higher than that of dogs, being on the average 9.2 mg. per 100 cc. The results of twenty-eight analyses of dog's blood are given by the following figures, which are arranged in ascending order.

Sample No.	Amino-acid nitrogen.	Sample No.	Amino-acid nitrogen.	Sample No.	Amino-acid nitrogen.
	mg.		*mg.*		*mg.*
1	6.33	11	7.03	21	7.66
2	6.34	12	7.05	22	7.71
3	6.38	13	7.07	23	8.14
4	6.39	14	7.08	24	8.40
5	6.42	15	7.31	25	8.46
6	6.49	16	7.39	26	8.51
7	6.51	17	7.43	27	8.61
8	6.62	18	7.66	28	8.79
9	6.62	19	7.66		
10	6.90	20	7.66	Average..	7.31

Most of our experiments on dogs were carried out under anesthesia with morphine or other narcotics, so that we examined first whether or not anesthesia tends to change the content of amino-acid nitrogen in the blood. From Table I, it is obvious that anesthesia has no influence upon the amino-acid nitrogen content of the blood, though there was always more or less increase of blood sugar.

For rabbit's blood the following results were obtained.

Sample No.	Amino-acid nitrogen.	Sample No.	Amino-acid nitrogen.	Sample No.	Amino-acid nitrogen.
	mg.		mg.		mg.
1	7.22	13	8.74	25	9.92
2	7.40	14	8.74	26	9.97
3	7.60	15	8.97	27	9.97
4	8.11	16	9.04	28	10.05
5	8.21	17	9.06	29	10.18
6	8.30	18	9.11	30	10.18
7	8.38	19	9.38	31	10.21
8	8.42	20	9.64	32	10.37
9	8.48	21	9.71	33	10.57
10	8.48	22	9.73	34	10.58
11	8.55	23	9.73	35	10.60
12	8.64	24	9.76		
				Average..	9.20

TABLE I.

The Relation between Anesthesia and Amino-Acid Nitrogen Content of the Blood of Dogs.

No.	Body weight.	Narcotic.	Before or after anesthesia.	After anesthesia.	Blood sugar.	Amino-acid nitrogen per 100 cc.
	kg.			hrs.	per cent	mg
1	5.0	Chloroform.	Before.		1.40	6.90
			After.	3	2.06	6.90
2	5.4	Morphinæ hydrochloridum hypodermically, 0.25 gm.	Before.		1.62	6.57
			After.	3	2.25	6.92
3	15.2	Morphinæ hydrochloridum hypodermically, 0.3 gm.; and ether insufflation.	Before.		1.25	8.61
			After.	1	1.80	8.61
			"	3	2.00	8.42
4	17.7	Ether insufflation.	Before.		1.10	7.96
			After.	1	1.90	8.14
			"	3	1.92	8.14
5	8.5	Morphinæ hydrochloridum hypodermically, 0.26 gm.	"	0.25	1.60	7.72
			"	4	2.08	7.72
			"	6	2.30	7.63
			"	8	2.00	7.83
6	11.5	Morphinæ hydrochloridum hypodermically, 0.2 gm.; and injection of 30 cc. of blood into the peritoneal cavity.	"	0.5	1.13	8.12
			"	4	1.16	8.12
			"	6	1.18	8.12
			"	8	1.34	8.51

In Experiment 6 we injected the blood of the same animal into the peritoneal cavity to see whether any change in the amino-acid content of the blood would result, as some of our experiments

TABLE II.

The Influence of the Removal of the Pancreas on the Sugar and Amino-Acid Nitrogen Content of the Blood of Dogs.

No.	Body weight.	Morphine injected.	Before or after removal of pancreas.	Time after removal.	Blood sugar.	Amino-acid nitrogen per 100 cc.
	kg.	*gm.*		*hrs.*	*per cent*	*mg.*
1	18.5	0.25	Before.		1.23	6.51
			After.	4	1.72	7.93
2	8.75	0.2	Before.		1.15	7.71
			After.	4	2.28	9.09
			"	6	2.50	9.70
3	13.0	0.25	Before.		1.94	9.08
			After.	4	2.41	10.75
			"	6.5	2.74	13.26
4	20.8	0.3	Before.		1.92	29.45
			After.	4	2.77	35.45
			"	6	2.97	39.05
			"	8	3.15	40.49
5	11.0	0.11	Before.		1.35	6.34
			After.	4	2.30	7.84
			"	6	2.56	8.54
			"	8	2.74	9.65
6	9.5 (Sugar in urine, 3.6 per cent).		Before.		0.98	7.94
			After.	22	2.83	12.76
7	10.1 (Sugar in urine, 3.3 per cent).		Before.		1.05	7.49
			After.	28	4.41	14.35

involve bleeding in the peritoneal cavity. The result shows that there is no influence.

The close relationship between the blood sugar content and the functions of endocrine glands such as pancreas, suprarenals,

thyroid, etc., is well known.   It seems, therefore, interesting to investigate whether the amino-acid content of the blood has any relation to the functions of such endocrine glands.   First we removed the pancreas of dogs and observed the influence.

The complete removal of the pancreas was established after each experiment.   The removal of the pancreas causes an increase of amino-acid in the blood as well as of blood sugar.   Whether this result may be of transient character or permanent will be seen in Table III.

The above experiments show us that the increase of amino-acid nitrogen content in the blood is still remarkable 2 days after the thorough removal of the pancreas, while thereafter it diminishes again even to the subnormal amount.   The blood sugar shows continually its high rate of percentage, though the excretion of sugar in urine diminishes after a while.   When a part of the pancreas is left under the skin, no increase of amino-acid nitrogen or of sugar occurs.   The change in percentage of urea nitrogen and of non-protein nitrogen in the blood is inconstant.

Adrenalin was found to be without effect on the amino-acid content of blood.

Five rabbits each received hypodermically 1 cc. of 1 : 1,000 adrenalin solution per kilo.   The amino-acid nitrogen figures before and 2 to 3 hours after were respectively 9.11 and 7.11, 10.05 and 10.05, 8.38 and 8.35, 7.40 and 7.23, 8.74 and 8.74 mg. per 100 cc. of blood.

Similarly, negative results were obtained in eight experiments in which pituitrin was injected.

It is well known that the thyroid gland is essentially important to metabolism.   Bock reported a case of hyperthyroidism showing an increase in blood amino nitrogen over the normal.   We examined five cases of hyperthyroidism (Graves' disease) and found that all of them were of normal amino-acid content.   We also extirpated the thyroid glands of four dogs without effect on the blood amino nitrogen.   In one case the parathyroids also were removed so that the animal died of tetany 5 days after the operation.   In the other cases no tetany occurred.   We may therefore conclude that the thyroid gland has no influence upon the amino-acid nitrogen content of the blood.

TABLE III.

Experiments on the Removal of the Pancreas of Dogs.

No.	Date.	Body weight	Operation.	Before or after operation.	Time after operation.	Blood sugar.	Amino-acid nitrogen per 100 cc. of blood.	Urea nitrogen per 100 cc. of blood.	Non-protein nitrogen per 100 cc. of blood.	Remarks.
	1918	*kg.*			*days*	*per cent*	*mg.*	*mg.*	*mg.*	
1	July 10	7.4	A part of the pancreas was left under the skin. Pancreas thoroughly removed.	Before.		1.53	6.33	13.05		
	" 15			After.	5	1.43	6.46	13.28		
	" 19									
	" 22			After reoperation.	3	3.75	8.37	13.28		Sugar in urine, 4.2 per cent.
2	" 20	11.4	Pancreas thoroughly removed.	Before.		1.74	7.31	13.98	47.76	
	" 27			After.	7	4.30	8.79	12.58	40.73	Sugar in urine, 10.5 per cent.
	Aug. 2			"	13	3.84	7.02	13.05	38.83	Sugar in urine, 1.9 per cent.
	" 6			"	17	4.00	5.90	10.72	50.74	
3	" 1	15.5	Pancreas thoroughly removed.	Before.		1.66	7.39	10.25	44.35	
	" 3			After.	2	3.75	11.90	15.38	60.88	
4	" 10	7.5	Pancreas thoroughly removed.	Before.		2.85	6.49	10.72	33.04	
	" 12			After.	2	3.66	9.11	12.35	50.09	
	" 16			"	6	3.12	6.28	10.25	39.92	
	" 23			"	13	3.22	4.81	10.49		Sugar in urine, 7.2 per cent.

	Date									Remarks
5	Sept. 3 " 5	12.0	Pancreas thoroughly re-moved.	Before. After.	2	2.44 4.65	7.05 13.11	11.65 24.23		Died of acute peritonitis.
6	" 10 " 12	11.5	Pancreas thoroughly re-moved.	Before. After.	2	1.77 3.33	7.07 10.62	10.72 16.31		Sugar in urine, 9.8 per cent.
7	" 30 Oct. 2	12.4	Pancreas thoroughly re-moved.	Before. After.	2	2.22 4.44	7.03 13.14	10.72 19.11	52.79 61.42	
8	" 23 " 25	11.5	Pancreas thoroughly re-moved.	Before. After.	2	2.32 3.61	6.39 8.99	11.65 23.07	64.70 67.50	
9	Dec. 11 " 13 " 13	7.5	A part of the pancreas was left under the skin. Pancreas thoroughly re-moved. After reoperation.	Before. After.	2	2.51 1.18 3.34	5.76 6.32 8.09	11.18 12.12 12.58	26.12 42.30 44.23	Sugar in urine, 7.8 per cent, by autopsy no peritonitis.

The effect of kidney extirpation on the amino-acid nitrogen content of the blood was also studied.   It is well known that when kidneys are extirpated or the ureters are ligated, a pronounced increase of urea and non-protein nitrogen in the blood will occur.   Bock investigated pathological bloods and found the most pronounced variations from the normal in nephritis.   We have confirmed Bock in observations on patients with uremia and

TABLE IV.

Experiments on the Removal of the Thyroid Gland of Dogs.

No.	Date.	Body weight.	Before or after extirpation.	Time after extirpation.	Blood sugar.	Amino-acid nitrogen per 100 cc.	Urea nitrogen per 100 cc. of blood.
	1918	*kg.*		*days*	*per cent*	*mg.*	*mg.*
1	Aug. ·29	12.0	Before.		1.69	8.46	10.49
	" 31		After.	2	1.53	7.80	10.25
2	Sept. 14	16.2	Before.		1.37	8.79	11.18
	" 16		After.	2	2.00	7.05	10.72
	" 21		"	7	2.30	6.72	11.18
	" 30		"	16	1.70	6.71	9.79
	Oct. 7		"	23	2.12	6.61	10.25
3	Sept. 21	16.5	Before.		1.46	6.42	11.65
	" 23		After.	2	2.00	6.19	10.25
	" 28		"	7	1.98	5.72	11.18
4	Oct. 31	12.0	Before.		1.98	7.66	10.25
	Nov. 2		After.	2	1.52	7.31	10.65
	" 12		"	12	1.57	6.71	9.79
	Dec. 13		"	43	0.98	7.65	10.72
	1919						
	Jan. 10		"	71	1.02	7.37	9.79
	Feb. 13	15.7	"	105	1.15	7.05	10.72

severe nephritis, and have also found a marked increase of amino-acid nitrogen in the blood of dogs after ligating the ureters or extirpating the kidneys.

Pilocarpin, as is well known, is an excitant for the secretion of the salivary and lachrymal glands, the mucous glands of the mouth, throat, nose, and deeper respiratory passages, the gastric secretory glands, the pancreas, and the intestinal glands.   It also

excites the suprarenal bodies and causes their increased intestinal secretion and influences the nervous control of glycogen in the liver.   The amylase and the maltase in the blood will copiously increase as in the case of the ligature of the pancreatic duct. It seems reasonable, therefore, to try to find out whether such a drug also has any influence upon the amino-acid content of the blood.   The result of the hypodermical injections of pilocarpin hydrochloricum to five dogs tells us that it causes a definite increase of the amino-acid content in the blood (35 to 65 per cent).

TABLE V.

Experiments on the Removal of Kidneys or the Ligature of Ureters on Dogs.

No.	Date.	Body weight.	Operation.	Before or after operation.	Time after operation.	Blood sugar.	Amino-acid nitrogen per 100 cc.	Urea nitrogen per 100 cc. of blood.	Non-protein nitrogen per 100 cc.
	1918	*kg.*			*days*	*per cent*	*mg.*	*mg.*	*mg.*
1	May 24	9.8	Ligature of both ureters.	Before.		1.91	6.42	23.77	
	" 26			After.	2	2.10	16.44	131.09	
2	June 2	17.7	Both ureters cut off.	Before.		1.74	7.08	11.65	42.24
	" 4			After.	2	1.57	13.17	67.07	157.98
	" 5			"	3	1.92	17.82	181.74	226.09
3	" 24	21.2	Both ureters cut off.	Before.		1.37	6.62	12.58	56.89
	" 25			After.	1¼	1.26	11.71	44.12	100.65
4	July 1	9.5	Extirpation of both kidneys.	Before.		1.38	7.43	24.32	70.11
	" 3			After.	2	1.50	15.31	115.57	224.61

We have determined the blood amino nitrogen in a large number of ward cases, with consistently abnormal results only in advanced nephritis and leucemia.   Since the results in leucemia appear to be new, we give them in Table VII.

From Table VII, it will be seen that the amino-acid nitrogen content of the blood increases or decreases in a fairly constant proportion to the number of white corpuscles.   X-ray treatment decreases the amino-acid nitrogen content as well as the number of white corpuscles, while it increases the red corpuscles in the

blood. Therefore, the red blood cells do not seem so essential
a factor for the amino-acid content in the blood as the white
blood cells. To ascertain more exactly the relationship between
white cells and amino-acids we divided the blood into fractions

TABLE VI.

*Experiments of the Hypodermic Injections of Pilocarpin Hydrochloricum
to Dogs.*

No.	Body weight	Pilocarpin injected.	Before or after injection.	Time before or after injection.	Blood sugar	Amino-acid nitrogen per 100 cc.
	kg.	*gm.*		*hrs.*	*per cent*	*mg.*
1	13.9	0.14	Before.		2.82	7.55
			After.	2	3.75	10.51
			"	4	1.90	8.21
2	7.7	0.08	Before.	2	1.66	7.17
			"		1.96	7.17
			After.	2	2.00	11.96
			"	3	1.33	11.27
3	17.8	0.18	Before.	1	2.50	6.38
			"		2.50	6.54
			After.	2	3.75	8.83
	.		"	4	1.53	7.38
			"	5.7	1.56	8.58
4	˙9.2	0.095	Before.	1	2.98	8.13
			"		3.07	6.83
			After.	1	4.00	11.06
			"	2	4.29	10.09
			"	3	2.50	11.39
5	7.5	0.075	Before.	1	1.60	7.60
			"		1.77	7.60
			After.	1	1.75	10.03
			"	2	1.51	10.64
			"	3	1.41	11.01

and determined the amino-acid content in each fraction. The
results show that the white blood cells contain six or seven times
as much amino-acid as the plasma. The red and white blood cells
cannot be separated perfectly from each other except by means
of high speed centrifuge, so that the relatively high content of

amino-acid in the red blood cells seems mainly due to the mixing of white blood cells.

TABLE VII.

Amino-Acid Nitrogen and Corpuscles in the Blood by Leucemia.

Sample No.	Diagnosis.	Amino-acid nitrogen per 100 cc.	No. of white corpuscles.	No. of red corpuscles.
		mg.	c. mm.	c. mm.
4	Chronic myeloid leucemia.........	11.27	212,600	
31	Subacute lymphoid "	6.11	4,000	1,040,000
32	Chronic myeloid leucemia.........	12.86	244,500	4,780,000
	Same after x-ray treatment	8.60	35,310	5,032,000
51	Chronic myeloid leucemia	17.81	286,100	3,520,000
	Same after x-ray treatment	6.68	13,800	4,185,000
52	Chronic myeloid leucemia.........	29.22	582,400	2,840,000
	Same after x-ray treatment	19.24	399,000	2,688,000
55	Chronic myeloid leucemia	13.14	205,800	3,520,000
56	Acute lymphoid "	5.60	1,000	886,000
58	Subacute myeloid "	8.02	64,000	852,480
59	Chronic myeloid "	12.12	132,600	3,766,400
61	Acute lymphoid "	14.16	507,200	3,500,000

TABLE VIII.

Amino-Acid Nitrogen Content in the Fractions of the Blood of Leucemia.

Sample No.	Whole blood.	Per 100 cc. of blood.		
		Red corpuscles.	White corpuscles.	Plasma.
		mg.	mg.	mg.
51	17.81		32.88	5.18
55	11.77	17.40	37.57	4.80

Bock has shown that red blood cells of birds are exceptionally rich in amino-acids. These cells, like human leucocytes, are characterized by the fact that they contain nuclei. Unfortunately

we are not able to separate nuclei from protoplasm without changing the amino-acid content in them, so that the conclusion may not be definitely established that it is nucleus which contains amino-acid in exceptional concentration in the human white corpuscles and in the bird red blood cells. A suggestive fact is observed, however, in the analysis of birds' eggs. Here the yolk corresponds to the nucleus, the white to the protoplasm. We found that the yolk of eggs of domestic fowls contained 32.79 mg. of amino-acid nitrogen per 100 cc., while the white contained only 6.49 mg.

<div align="center">SUMMARY.</div>

1. The amino-acid nitrogen content of the blood of twenty-eight fasting dogs per 100 cc. was found to vary from 6.33 to 8.79 mg.; that of rabbits is somewhat higher, from 7.22 to 10.60 mg.

2. Anesthesia does not influence the amino-acid nitrogen content of the blood during the course of 8 hours.

3. The thorough removal of the pancreas causes a transient increase of the amino-acid nitrogen in the blood; the increase begins within a few hours and lasts for at least 2 days after extirpation, after which it diminishes again even to a subnormal figure. When a part of the pancreas is left under the skin in connection with the blood vessels, the blood amino nitrogen remains normal.

4. Hypodermic injection of neither adrenalin nor pituitrin influences the amino-acid nitrogen content of the blood.

5. Neither the removal of the thyroid gland nor hyperthyroidism influences the amino-acid nitrogen content of the blood.

6. The ligature of both ureters or the extirpation of both kidneys causes a remarkable increase of the amino-acid nitrogen, in parallelism with the increase of urea and non-protein nitrogen in the blood.

7. Hypodermic injection of pilocarpin causes an increase of the amino-acid nitrogen content in the blood.

8. The amino-acid nitrogen content in the blood is increased in leucemia. The increase parallels the number of white corpuscles.

9. The analysis in fractions of the blood of leucemia shows that the white blood cells contain six or seven times as much amino-acid nitrogen as the plasma. It is suggestive that the nuclei

of the white corpuscles are the essential part for containing such a high rate of amino-acid, though the conclusion is not definitely established.

It is a pleasure, as well as a duty, to acknowledge our indebtedness to Professor R. Inada for his constant inspiration and courtesy during this work.

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THE HYDROGEN ION CONCENTRATION OF THE INTESTINAL CONTENTS.

By SEIZABURO OKADA and MINORU ARAI.

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The intestinal juice has been regarded as distinctly alkaline. Auerbach and Pick (1) examined the intestinal juice of dogs and found the hydrogen ion concentration to be 0.5 to 5.0×10^{-8}, but, allowing for errors in the method, they estimated that the real reaction of the intestinal juice was 2×10^{-8}. This reaction coincides with that of pancreatic juice estimated by them and with that of bile from the liver determined by one of us (2). It also corresponds fairly well to the optimal reaction of trypsin estimated by Meyer (3) and Michaelis and Davidsohn (4), to that of erepsin shown by Rona and Arnheim (5), and to that of the lipase of pancreatic juice found by Davidsohn (6). McClendon (7) determined the human adult duodenal contents taken with the duodenal tube and found most cases to be very near to 2×10^{-8}. He also examined the duodenal contents of sucklings and found them to be 4.0×10^{-3} to 1.5×10^{-7}, an average of 8×10^{-4}. He reports that when the stomach contents of the sucklings are strongly acid, the duodenal contents are less acid, but when the former are weakly acid, then the latter are more acid. McClendon, Shedlov, and Thomson (8) examined the ileal contents of seven puppies from 4 to 46 days after birth and found the hydrogen ion concentration to be from 2×10^{-6} to 1.8×10^{-7}. McClendon, Shedlov, and Karpman (9) examined the contents of the ileum of four dogs from $2\frac{3}{4}$ to $4\frac{1}{2}$ hours after a mixed diet of cooked food, in two cases of which magnesium sulfate was also administered, and found the hydrogen ion concentration to be 2.5×10^{-6} to 2.5×10^{-7}. Long and Fenger (10), using the duodenal tube, observed a variation in the adult duodenum from pH 3.80 to 7.81. Myers and McClendon (11),

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TABLE I.

The Hydrogen Ion Concentration of the Duodenal Contents of Hospital Patients.

Sample No.	Sex	Age yrs.	Diagnosis.	Meal.	Time after meal. hrs.	Quality of the fluid.	Reaction to litmus paper.	pH	H+	Remarks.
1	♀	19	Progressive muscular dystrophy.	Fasting.		Yellow and clear.	Alkaline.	7.15	7.1×10^{-8}	
2	♂	24	Urticaria.	200 cc. of milk.	3	" " "	"	7.43	3.7×10^{-8}	Stomach juice, pH = 1.43
3	♀	23	Beri-beri.	Fasting.		" " a little turbid.	Acid.	6.59	2.6×10^{-7}	
4	♂	51	Pancreatitis.	3 cups of rice pap and 1 egg.	5½	Yellow and somewhat turbid.	Neutral.	6.85	1.4×10^{-7}	Stomach juice, pH = 6.25
5	♀	33	Graves' disease.	Fasting.		Yellowish and clear.	Alkaline.	7.50	3.2×10^{-8}	Stomach juice, pH = 4.37
6	♂	44	Achylia gastrica.	Ewald-Boas test meal.	3	Yellowish and clear.	"	7.97	1.1×10^{-8}	Stomach juice, pH = 6.62
7	♀	23	Disease of the spinal cord.	Fasting.		Gold yellow and clear.	"	7.27	5.4×10^{-8}	
8	♂	26	Pleurisy.	"		Gold yellow and clear.	"	7.38	4.2×10^{-8}	
9	♂	19	Beri-beri.	"		Gold yellow and clear.	"	7.76	1.7×10^{-8}	

No.		Diagnosis	Diet	Hrs.	Appearance	Reaction	pH	
10 ♂	29	Cerebral tumor.	Fasting.		Yellow and slimy.	Alkaline.	6.92	1.2×10^{-7}
			"		" " clear.	"	6.85	1.4×10^{-7}
			200 cc. of milk and 2 eggs.	2	" " a little turbid.	"	7.00	1.0×10^{-7}
			200 cc. of milk and 2 eggs.	3½	Yellow and turbid.	Acid.	6.05	8.9×10^{-7}
11 ♂	55		Usual diet.	6	" " somewhat turbid.	Alkaline.	7.84	1.4×10^{-8}
12 ♂	35	Tabes dorsalis.	Fasting.		Yellow and a little turbid.	"	7.14	7.2×10^{-8}
			200 cc. of milk and 2 eggs.	½	Yellow and severely turbid.	Acid.	5.97	1.1×10^{-6}
			200 cc. of milk and 2 eggs.	1	Yellow and severely turbid.	"	5.48	3.3×10^{-6}
			200 cc. of milk and 2 eggs.	1½	Yellow and turbid.	"	4.80	1.6×10^{-6}
			200 cc. of milk and 2 eggs.	2	" " "	"	4.83	1.5×10^{-5}
13 ♂	64	Diabetes mellitus.	Fasting.		Yellowish and clear.	Alkaline.	7.90	1.3×10^{-8}
14 ♂	21	Ancylostomiasis.	"		"	"	7.52	3.0×10^{-8}
			200 cc. of milk and 2 eggs.	3	Yellow and clear.	Acid.	6.65	2.2×10^{-7}
15 ♂	22	Valvular disease of the heart.	200 cc. of milk and 2 eggs.	3	Yellow and clear.	Neutral.	7.05	8.9×10^{-8}

applying the same method to one of themselves, found the reaction of the duodenum between 3 and 4 hours after various meals to be from pH 3.20 to 7.82.

Michaelis and Mendelssohn (12) found the reaction of feces in the adult was 1×10^{-8}, in the suckling 1×10^{-6} to 1×10^{-7}; while Howe and Hawk (13) found a hydrogen ion concentration of 0.15 to 9.8×10^{-8} in the adult.

We examined electrometrically the duodenal contents of various hospital patients, removed with an Einhorn duodenal tube under

TABLE II.

The Hydrogen Ion Concentration of the Intestinal Contents of Dogs.

Sample No.	Body weight.	Sex.	Meal.		Time after meal.	Parts of the intestine.	Reaction to litmus paper.	pH	H+
	kg.				*hrs.*				
1	6.6	♀	1 lb. meat.		3	Last part of ileum.	Alkaline.	6.78	1.7×10^{-7}
2	16.7	♂	1 " "		1	Last part of duodenum.	"	7.17	6.8×10^{-8}
3	15.5	♂	1 " "		2	Last part of duodenum.	"	7.68	2.1×10^{-8}
			1 " "		2	Last part of ileum.	Acid.	6.59	2.6×10^{-7}
4	6.1	♀	1 " "		2	Last part of duodenum.	"	6.15	7.2×10^{-7}
			1 " "		2	Last part of ileum.	Alkaline.	7.83	1.5×10^{-8}
5	Pup of 25 days, whose stomach was filled with coagulated milk; the pH of which was 3.82, the pH of the ileum was 5.92.								

various conditions, and the duodenal and ileal contents of dogs taken out directly. The results obtained will be seen in Tables I and II.

In fasting, the duodenal contents are mainly composed of a mixture of bile and intestinal and pancreatic juices. That both the latter juices are alkaline has been described above. The bile from the liver is also alkaline, but this is mainly poured into the duodenum by the time of digestion and only the bile from the gall bladder will be occasionally poured into it. The reaction of the

bile from the gall bladder is variable; it may be acid, neutral, or alkaline.   The hydrogen ion concentration was found by Okada (2) to be from 4.7×10^{-6} to 3.4×10^{-8}.   The reaction of the duodenal contents may not logically surpass the limit of the reactions of these fluids.   Of the eleven cases examined by us for the duodenal contents when fasting, we found eight cases to be alkaline and three cases acid, the hydrogen ion concentration of which was from 2.6×10^{-7} to 1.3×10^{-8}.   By the time of digestion the acid chyme may be poured into the duodenum and the secretion of the digesting juices may be copious and in pathological cases the action of microorganisms must also be accounted for, so that the relation is somewhat confused.   Of the twelve cases examined we found that seven cases were acid, four cases alkaline, and one case was neutral, the hydrogen ion concentration being from 1.6×10^{-5} to 1.1×10^{-8}.   The tests for free hydrochloric acid were always negative.   No special relation seems to exist between the acidity of the stomach and the reaction of the duodenal contents.

The reaction of the duodenal or the ileal contents examined in four dogs was acid or alkaline.   On the reaction of ileal contents, the action of the microorganism seems especially to dominate. One puppy was examined and the ileal contents were found to be slightly acid.

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THE INFLUENCE OF PUTREFACTION PRODUCTS ON CELLULAR METABOLISM.

II. ·ON THE INFLUENCE OF PHENYLACETIC AND PHENYL-PROPIONIC ACIDS ON THE DISTRIBUTION OF NITROGEN IN THE URINE.

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The investigations of H. and E. Salkowski[1] have established the fact that in the putrefaction of proteins, aside from other products, two are formed which are homologous with benzoic acid, namely, phenylacetic and phenylpropionic acids; and that these acids when introduced into mammals, reappear in the urine conjugated with glycocoll, the phenylacetic as phenaceturic[2] and the phenylpropionic, having been oxidized to benzoic, as hippuric acid. It is possible, of course, that putrefaction of protein material in the intestinal canal may give rise to the acids investigated and thus account for the occurrence of phenaceturic and hippuric acids in normal urine. Unfortunately no attempts have thus far been successful in establishing directly the presence of either phenyl-acetic or phenylpropionic acid in the intestinal contents or excre-

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[1] Salkowski, E., *Z. physiol. Chem.*, 1878–79, ii, 420; 1883–84, viii, 149; 1885, ix, 229, 491. Salkowski, E., and Salkowski, H., *Z. physiol. Chem.*, 1882–83, vii, 161, 169; *Ber. chem. Ges.*, 1879, xii, 107.

[2] Recently, Thierfelder and Sherwin reported that, after administering phenylacetic acid, there appeared in the urine of man, not phenylaceturic (as in the case of dogs and rabbits) or phenylacetornithuric acid (as with birds) but phenacetylglutamic acid which could be isolated, partly combined with urea and partly uncombined (Thierfelder, H., and Sherwin, C. P., *Ber. chem. Ges.*, 1914, xlvii, 2630–34; *Jahresb. Thierchem.*, 1914, xliv, 1058; *Z. physiol. Chem.*, 1915, xciv, 1).

ment,[3] possibly, owing to the difficulty of isolating small quantities of these acids from such mixtures.

Several studies have been made of the influence of the acids on the excretion of nitrogen in the urine. E. Salkowski[4] has shown that, on feeding phenylacetic acid to rabbits, the nitrogen excreted in the urine is increased. Desgrez and Guende[5] have reported that phenylpropionic acid causes a decrease in the nitrogen in the urine when fed to guinea pigs; the decrease being due to a smaller excretion of urea. This leads to the conclusion that two homologous aromatic acids, which probably arise from the putrefaction of protein material in the intestine and which can combine with the same nitrogen-containing conjugate, glycocoll, are playing quite opposing rôles in the excretion of nitrogen in the urine. If we assume with Salkowski and others[6] that the introduction of benzoic acid causes a marked increase in the decomposition of tissue proteins, the same effect must also be ascribed to phenylpropionic acid, since it is oxidized in the animal body to benzoic acid. Contrary to Desgrez and Guende's statement, therefore, introduction of the latter substance should cause an increase in the nitrogen excreted in the urine just as in the case of phenylacetic acid.

The assumption is justified by the results of the following experiments which were carried out in the manner described in an earlier paper.[7] A number of the rabbits used were maintained in N balance by feeding "tofukara,"[8] while others were fasted. The total nitrogen was determined by the Kjeldahl method, the urea by means of urease, the ammonia by the method of Krüger, Reich, and Schittenhelm, and the amino-acids by Van Slyke's method.

[3] Tappeiner has shown the presence of phenylpropionic acid in the paunches of ruminants fed with hay; but it is uncertain whether the acid existed preformed in the hay or was formed in the alimentary canal from protein (Tappeiner, H., *Z. Biol.*, 1886, xxii, 236–40).

[4] Salkowski, E., *Z. physiol. Chem.*, 1888, xii, 222.

[5] Desgrez and Guende, *Compt. rend. Soc. biol.*, 1906, lviii, 526.

[6] Salkowski, E., *Z. physiol. Chem.*, 1877–78, i, 1; *Virchows Arch. path. Anat.*, 1888, cxiii, 394. Virchow, *Z. physiol. Chem.*, 1877–78, i, 78. Kumagawa, M., *Virchows Arch. path. Anat.*, 1888, cxiii, 134.

[7] Hijikata, Y., Acta scholae medicinalis universitatis imperialis in Kyoto, iv.

[8] Tofukara, a waste product obtained in the preparation of bean cheese, was pressed daily and a weighed quantity soaked in a fixed amount of water.

Experiments with Phenylacetic Acid.

Experiment 1.—A 1,900 gm. rabbit, in N balance, received on Oct. 30, 0.5 gm. of the acid[a] mixed with his food and on the following day, 1.0 gm. In both administrations the acid was neutralized with sodium carbonate solution.

On both days in which the acid was given in the food, the excretion of amino-acids increased strikingly, while that of the total nitrogen was unchanged, causing a considerable increase in the proportion of the former to the latter. The excretion of ammonia was unaltered. A small decrease in the ratio of urea to total nitrogen is mainly due to an insignificant decrease in the absolute quantity of urea. The fact that there was no increase in the total nitrogen excreted, was to be expected since the amount of acid administered was too small to cause any decomposition of tissue proteins.

Experiment 2.—A rabbit, weighing 2,420 gm. and in N balance, received on both Nov. 13 and 14, by means of the stomach tube, 1.2 gm. of phenylacetic acid, dissolved in sodium carbonate solution.

It is apparent that on both days in which the acid was given, not only the output of amino-acids, but also those of total nitrogen, urea, and ammonia were increased, in spite of a considerable interference with the appetite. While the relative quantity of urea was very slightly decreased, the relative quantities of ammonia and amino-acids were more than doubled. The experiment was ended when the animal refused to take any more food.

Experiment 3.—The rabbit weighed 2,830 gm. Until fasting was begun, it received tofukara; then 35 cc. of water once daily through the stomach tube. On both the 4th and 5th days of fasting, it received 1.0 gm. of phenylacetic acid, neutralized with 35 cc. of sodium carbonate solution through the stomach tube.

The total nitrogen excretion decreased during fasting until the acid was administered. There was then an increase for 2 days

[a] The phenylacetic acid was prepared by Totani, according to Mann, and was analytically pure. It was identical with that used in his experiments reported in his famous work, "Über das Verhalten der Phenylessigsäure im organismus des Huhns." I take this occasion to express to him my thanks for his courtesy in supplying it (Totani, G., *Z. physiol. Chem.*, 1910, lxviii, 75; Mann, W., *Ber. chem. Ges.*, 1881, xiv, 1645).

TABLE I.

Date.	Body weight.	Volume of urine.	Specific gravity.	Total N.	Urea N.		Ammonia N.		Amino-acid N.		Remarks.
	gm.	cc.		mg.	mg.	per cent total N	mg.	per cent total N	mg.	per cent total N	
1918											
Oct. 26	1,900	170	1.020	1,597.1	1,413.3	88.47	1.2	0.08	12.8	0.80	
" 27	1,900	165	1.019	1,585.9	1,410.5	88.94	1.7	0.11	12.9	0.81	
" 28	1,900	160	1.020	1,529.9	1,358.0	88.76	1.5	0.10	11.6	0.76	
" 29	1,910	165	1.020	1,670.0	1,500.2	89.83	1.7	0.10	14.1	0.84	
" 30	1,920	150	1.020	1,529.9	1,309.6	85.60	1.7	0.11	31.0	2.03	0.5 gm. phenylacetic acid in the diet.
" 31	1,930	140	1.021	1,602.7	1,354.6	84.52	1.6	0.10	55.4	3.46	1.0 " " " "
Nov. 1	1,940	155	1.020	1,557.9	1,382.9	88.77	1.3	0.08	14.9	0.96	
" 2	1,930	160	1.018	1,602.7	1,405.3	87.68	1.3	0.08	11.7	0.73	
" 3	1,930	165	1.018	1,608.4	1,438.7	89.45	1.5	0.09	12.1	0.75	
" 4	1,930	150	1.020	1,608.4	1,410.5	87.70	1.7	0.11	17.1	1.06	

TABLE II.

Date.	Body weight.	Volume of urine.	Specific gravity.	Total N.	Urea N.		Ammonia N.		Amino-acid N.		Remarks.
	gm.	cc.		mg.	mg.	per cent total N	mg.	per cent total N	mg.	per cent total N	
1918											
Nov. 9	2,420	200	1.018	1,961.4	1,729.4	88.17	2.2	0.11	23.2	1.18	
" 10	2,420	200	1.020	2,095.9	1,855.9	88.55	2.4	0.12	23.8	1.14	
" 11	2,440	240	1.016	2,084.7	1,848.5	88.67	3.1	0.15	23.4	1.12	
" 12	2,440	195	1.019	2,020.2	1,794.4	88.87	4.5	0.22	22.6	1.12	
" 13	2,420	145	1.031	2,869.3	2,516.7	87.71	16.3	0.57	74.9	2.61	1.2 gm. phenylacetic acid through stomach tube. ⅓ of this day's ration not eaten.
" 14	2,380	225	1.021	3,205.5	2,831.0	88.03	10.2	0.32	81.0	2.52	1.2 gm. phenylacetic acid through stomach tube. ⅔ of this day's ration not eaten.
" 15											Nothing eaten.

TABLE III.

35 Cc. Distilled Water through Stomach Tube Each Day.

Day of eating.	Body weight. gm.	Volume of urine. cc.	Specific gravity.	Reaction.	Total N. mg.	Urea N.		Ammonia N.		Amino-acid N.		Remarks.
						mg.	per cent total N	mg.	per cent total N	mg.	per cent total N	
	2,830	255	1.020	Alkaline.	3,166.3	2,935.4	92.78	6.7	0.21	26.5	0.84	Last day of feeding.
1	2,720	75	1.035	Acid.	1,782.1	1,597.1	89.06	4.5	0.25	20.1	1.13	
2	2,650	48	1.045	"	1,597.1	1,407.9	88.15	4.3	0.27	18.4	1.15	
3	2,580	45	1.042	"	1,352.0	1,177.1	87.06	3.4	0.25	11.0	0.81	
4	2,510	47	1.048	"	1,513.1	1,249.7	82.59	5.6	0.37	91.5	6.44	
5	2,420	87	1.043	"	2,589.0	2,187.8	84.51	9.0	0.35	66.2	2.56	1.0 gm. phenylacetic acid through stomach tube.
6	2,320	70	1.036	"	1,894.2	1,665.2	87.91	4.8	0.22	34.5	1.82	" " "
7	2,220	63	1.041	"	2,023.0	1,797.8	88.87	6.7	0.33	23.0	1.15	" " "
8	2,150	98	1.041	"	3,283.9	2,947.7	89.76	16.8	0.51	27.0	0.82	

followed by a decrease. The outputs of urea, ammonia, and amino-acids showed increases; the urea absolutely and the others both relatively and absolutely.

Experiment 4.—The rabbit, weighing 3,130 gm., was fed tofukara until the beginning of the experiment, after which it received neither food nor water. On both the 4th and 5th fasting days, it received 0.5 gm. of phenylacetic acid, twice daily (10 a.m. and 5 p.m.). The acid, neutralized with 10 cc. of sodium carbonate solution, was injected subcutaneously.

The results agree with those obtained in the preceding experiments in which the animal received the substance *per os.*

Experiments with Phenylpropionic Acid.

Experiment 5.—A rabbit, weighing 2,550 gm., was maintained in N balance. It was given phenylpropionic acid[10] during two periods of 2 days each. The acid was neutralized with sodium carbonate solution and introduced through the stomach tube. On both Dec. 15 and 16 the animal received 1.0 gm. of the acid. On both the 21st and 22nd of Dec., 1.5 gm. were administered.

The total nitrogen and the ammonia excreted were practically unchanged when the acid was introduced. The output of ammonia increased strikingly on both occasions, both absolutely and relatively. In the first case, urea decreased relatively and in the second both absolutely and relatively. The amino-acids were greatly increased.

Experiment 6.—A rabbit, weighing 2,560 gm., in N balance, was treated in the same manner as the preceding one but with larger doses. On Dec. 23 and 24, it received 2.5 gm. daily of neutralized acid and on the 28th and 29th, 3.5 gm. daily.

On the first day in which the acid was administered, the total nitrogen excreted was increased. On the second day, it was normal. There was, also, an absolute increase in the output of urea on the first day. Relatively, however, it was decreased on both days. Both absolutely and relatively the ammonia and amino-acid excretions were increased on both days on which acid was given. During the second period of feeding the acids, similar changes were observed but of greater degree.

[10] The acid was prepared by the reduction of cinnamic acid with sodium amalgam as described by Erlenmeyer and Alexejeff (Erlenmeyer and Alexejeff, *Ann. Chem.*, 1862, cxxi, 375.

TABLE IV.

Day of feeding.	Body weight.	Volume of urine.	Specific gravity.	Reaction.	Total N.	Urea N.		Ammonia N.		Amino-acid N.		Remarks.
	gm.	cc.			mg.	mg.	per cent total N	mg.	per cent total N	mg.	per cent total N	
1	3,130	255	1.013	Alkaline.	2,443.3	2,237.7	91.59	3.9	0.16	32.5	1.33	Last day of feeding.
2	3,040	60	1.035	Acid.	1,591.5	1,375.2	86.41	3.4	0.21	29.8	1.87	
3	2,950	48	1.042	"	1,529.9	1,331.4	87.03	2.4	0.16	28.2	1.91	
4	2,880	51	1.045	"	1,815.7	1,633.8	89.98	2.6	0.14	33.6	1.85	
	2,800	60	1.046	.	2,443.3	2,075.0	84.98	4.1	0.17	85.6	3.50	Twice (10 a.m. and 5 p.m.) 0.5 gm. phenylacetic acid, dissolved 10 cc. soda solution, injected hypodermically.
5	2,690	85	1.047	"	2,992.5	2,565.9	85.74	6.3	0.21	110.5	3.65	" "
6	2,580	76	1.040	"	2,325.7	2,014.0	86.55	3.4	0.15	38.5	1.66	
7	2,500	60	1.042	"	1,972.6	1,768.1	89.63	2.8	0.14	37.9	1.92	
8	2,410	64	1.047	"	2,400.8	2,148.0	89.05	3.9	0.16	32.5	1.35	
9	2,330	60	1.047	"	2,499.4	2,264.6	90.66	5.0	0.20	36.8	1.48	

TABLE V.

Date.	Body weight.	Volume of urine.	Specific gravity.	Total N.	Urea N.		Ammonia N.		Amino-acid N.		Remarks.
	gm.	cc.		mg.	mg.	per cent total N	mg.	per cent total N	mg.	per cent total N	
1918											
Dec. 11	2,550	210	1.018	1,989.4	1,796.1	90.28	2.8	0.14	14.9	0.75	
" 12	2,560	225	1.017	1,950.2	1,750.0	89.74	4.1	0.21	16.6	0.85	
" 13	2,560	205	1.019	1,894.2	1,687.9	89.11	4.5	0.24	17.7	0.93	
" 14	2,560	220	1.018	2,051.1	1,855.5	90.46	5.0	0.24	15.3	0.75	
" 15	2,570	210	1.019	2,017.4	1,767.0	86.59	3.9	0.19	69.9	3.47	1.0 gm. phenylpropionic acid through stomach tube.
" 16	2,570	205	1.018	2,028.6	1,775.7	87.53	4.5	0.22	75.2	3.71	" "
" 17	2,570	210	1.018	2,034.3	1,833.0	90.11	4.3	0.21	15.3	0.75	
" 18	2,570	220	1.017	1,950.2	1,741.2	89.29	5.3	0.27	14.9	0.76	
" 19	2,560	210	1.017	1,939.0	1,745.0	89.91	5.0	0.26	15.3	0.79	
" 20	2,560	220	1.016	1,939.0	1,765.3	91.04	5.6	0.29	14.7	0.76	
" 21	2,570	210	1.019	2,028.6	1,771.7	87.34	4.8	0.24	82.4	4.06	1.5 gm. phenylpropionic acid through stomach tube.
" 22	2,560	220	1.018	1,883.9	1,627.7	86.41	3.1	0.17	84.7	4.50	" "
" 23	2,560	240	1.016	1,927.8	1,746.3	90.58	2.2	0.11	14.1	0.73	
" 24	2,570	240	1.015	1,944.6	1,752.1	90.10	2.0	0.10	13.5	0.69	

TABLE VI.

Date.	Body weight.	Volume of urine.	Specific gravity.	Total N.	Urea N.		Ammonia N.		Amino-acid N.		Remarks.
	gm.	cc.		mg.	mg.	per cent total N	mg.	per cent total N	mg.	per cent total N	
1918											
Dec. 20	2,560	240	1.015	2,140.7	1,942.2	90.73	2.2	0.10	15.1	0.71	
" 21	2,560	220	1.017	2,006.2	1,796.7	89.56	2.2	0.11	14.5	0.72	
" 22	2,550	240	1.015	2,174.4	1,958.1	90.05	3.3	0.15	18.8	0.87	
" 23	2,560	220	1.019	2,499.4	2,091.7	83.72	4.2	0.17	136.1	5.45	2.5 gm. phenylpropionic acid through stomach tube.
" 24	2,550	230	1.020	2,079.1	1,715.4	82.54	5.0	0.24	108.3	5.21	" "
" 25	2,550	235	1.017	2,045.5	1,796.1	87.81	2.8	0.14	17.5	0.86	
" 26	2,550	235	1.016	2,017.4	1,807.7	89.61	2.4	0.12	17.6	0.87	
" 27	2,550	240	1.016	2,179.6	1,952.9	89.60	2.9	0.13	19.9	0.91	
" 28	2,550	190	1.028	2,348.1	1,961.5	83.54	10.9	0.46	171.9	7.32	3.5 gm. phenylpropionic acid through stomach tube.
" 29	2,520	225	1.025	2,678.7	2,217.4	82.78	7.4	0.27	221.9	8.29	" "
" 30	2,520	210	1.019	2,398.5	2,154.7	89.84	4.2	0.19	24.1	1.00	
" 31	2,530	245	1.015	2,185.6	1,958.6	89.61	2.8	0.14	19.9	0.91	

TABLE VII.

35 Cc. Distilled Water through Stomach Tube Daily.

Day of fasting.	Body weight.	Volume of urine.	Specific gravity.	Reaction.	Total N.	Urea N.		Ammonia N.		Amino-acid N.		Remarks.
	gm.	cc.			mg.	mg.	per cent total N	mg.	per cent total N	mg.	per cent total N	
	3,120	335	1.017	Alkaline.	3,328.8	3,055.3	91.78	4.5	0.14	36.4	1.09	Last day of feeding.
1	2,940	125	1.030	Acid.	2,818.8	2,523.5	89.52	3.9	0.14	34.7	1.23	
2	2,840	77	1.038	"	2,342.5	2,107.1	89.83	2.8	0.12	32.4	1.38	
3	2,740	74	1.035	"	2,213.6	1,975.4	89.24	2.8	0.13	28.8	1.30	
4	2,680	71	1.037	"	2,191.2	1,991.0	90.86	2.8	0.13	24.2	1.10	
5	2,530	165	1.034	"	2,891.7	2,378.6	82.26	8.7	0.30	241.9	8.36	3.0 gm. phenylpropionic acid through stomach tube.
6	2,370	163	1.033	"	3,082.2	2,527.4	82.00	16.8	0.55	218.9	7.10	" "
7	2,280	79	1.040	"	2,846.8	2,569.4	90.26	14.0	0.49	31.1	1.09	" "
8	2,180	91	1.041	"	3,440.7	3,147.4	91.18	12.3	0.36	31.1	0.90	
9	2,080	98	1.039	"	3,678.4	3,331.0	90.56	31.4	0.85	34.6	0.92	

TABLE VIII.

Day of fasting.	Body weight.	Volume of urine.	Specific gravity.	Reaction.	Total N.	Urea N.		Ammonia N.		Amino-acid N.		Remarks.
	gm.	cc.			mg.	mg.	per cent total N	mg.	per cent total N	mg.	per cent total N	
	2,610	260	1.010	Alkaline.	2,051.1	1,898.1	92.54	1.7	0.08	17.6	0.86	Last day of feeding.
1	2,480	70	1.025	Acid.	913.5	781.1	85.51	2.0	0.22	14.3	1.57	
2	2,370	60	1.028	"	885.4	768.4	86.80	2.8	0.32	11.0	1.24	
3	2,280	53	1.030	"	1,044.6	922.5	88.31	2.2	0.21	12.1	1.16	
4	2,160	90	1.033	"	1,479.5	1,213.9	81.57	2.2	0.15	109.4	7.39	Twice (10 a.m. and 5 p.m.) 1.0 gm. phenylpropionic acid dissolved in 20 cc. soda solution, injected hypodermically.
5	2,070	61	1.038	"	1,322.5	1,060.9	80.22	3.9	0.30	119.1	8.93	"
6	1,990	24	1.040	"	857.4	675.5	78.80	2.6	0.30	17.5	2.04	"
7	1,930	20	1.045	"	700.5	553.7	79.04	1.1	0.16	13.7	1.96	Found dead next day.

TABLE IX.

Day of fasting.	Body weight.	Volume of urine.	Specific gravity.	Reaction.	Total N.	Urea N.		Ammonia N.		Amino-acid N.		Remarks.
	gm.	cc.			mg.	mg.	per cent total N	mg.	per cent total N	mg.	per cent total N	
	3,210	225	1.010	Alkaline.	1,916.6	1,772.6	92.49	3.9	0.20	21.3	1.11	Last day of feeding.
1	3,190	87	1.026	Acid.	1,826.9	1,644.2	90.00	3.4	0.19	20.9	1.14	
2	3,100	49	1.038	"	1,479.5	1,292.5	87.36	2.5	0.17	16.9	1.14	
3	3,020	48	1.043	"	1,630.8	1,410.0	86.46	2.2	0.13	14.1	0.86	
4	2,890	115	1.029	"	1,838.1	1,453.6	79.81	3.4	0.18	181.2	9.86	Twice (10 a.m. and 5 p.m.) 1.2 gm. phenylpropionic acid, dissolved in 15 cc. soda solution, injected hypodermically.
5	2,790	110	1.038	"	2,202.4	1,804.1	81.92	5.0	0.23	218.4	9.92	"
6	2,710	52	1.039	"	1,972.6	1,756.1	89.02	3.6	0.18	27.0	1.37	"
7	2,650	42	1.043	"	1,670.0	1,471.6	88.12	2.3	0.14	23.6	1.62	"
8	2,580	41	1.042	"	1,698.0	1,533.3	90.33	2.2	0.13	23.1	1.36	

Experiment 7.—The rabbit, weighing 3,120 gm., was fed with tofukara for a time and then fasted. During the experiment it received 35 cc. of water daily. On both the 5th and 6th days, 3.0 gm. of the acid, in 35 cc. of sodium carbonate solution, were introduced through the stomach tube.

The total excretion of nitrogen decreased regularly until the acid was administered. It then increased, with the exception of the seventh day, until death. The urea, also, rose absolutely beginning with the fifth day, but on the 2 days in which the acid was given it decreased relatively. The ammonia excretion paralleled that of the total nitrogen. There was a great increase in the quantity of amino-acids both absolutely and relatively.

Experiment 8.—A fasting rabbit, weighing 2,610 gm., received, on the 4th and 5th days, two subcutaneous injections daily of 1.0 gm. each of phenylpropionic acid in 20 cc. of sodium carbonate solution.

On both days in which the acid was given, the total nitrogen, as well as the urea and amino-acids, was increased. Relatively, however, the urea decreased. On the second day, the absolute quantity of ammonia was increased but there was relatively no change. The increase in the amino-acid excretion was both absolute and relative.

Experiment 9.—The rabbit weighed 3,210 gm. On the 4th and 5th days of fasting, it received, twice daily, 1.2 gm. of phenylpropionic acid. The acid, neutralized with 15 cc. of sodium carbonate solution, was injected subcutaneously.

The result of the experiment was to confirm the foregoing ones.

DISCUSSION.

The experiments reported show that both substances under investigation have the same effect on the distribution of nitrogen in the urine. The effect of the phenylpropionic acid is perhaps smaller.

The total nitrogen, as well as the ammonia, shows no essential change with a small dose. An insignificant decrease is noted in the output of urea. No simultaneous decreases in the excretion of total nitrogen and urea, such as those reported by Desgrez and Guende, in the case of phenylpropionic acid, were observed. The amino-acids in the urine showed a very considerable increase, not

only absolutely but relatively. Although no attempt was made to isolate glycocoll, it seems quite probable that the increase may have been largely due to this substance in the form of a conjugate.

The fact that the increase in the amino-acids is accompanied by a decrease in urea suggests the possibility that the amino-acids may arise from urea or its antecedent. The results may be of importance in clarifying our conception of the formation of glycocoll in the animal body, no experimental reduction of the urea in the urine having been observed heretofore, on introducing benzoic acid or analogous substances.

When the dose was increased the total nitrogen excretion was, without exception considerably augmented, thus confirming the observations of Salkowski on phenylacetic acid. The increases in the absolute and relative quantities of ammonia and amino-acids are remarkably great. The urea also rises but not in proportion to the total nitrogen. The increase in the ammonia, even though the acids were neutralized, favors the view that the simultaneous increases in the quantities of total nitrogen and urea excreted may have been due to a pathological decomposition of tissue proteins. These changes were observed not only with rabbits maintained in N balance but with fasting animals.

ON THE CLEAVAGE PRODUCTS OF THE CRYSTALLINE LENS.

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Since the study of the hydrolysis of the proteins has shown that they are composed of various combinations of certain substances, which, for given bodies, are united in characteristic proportions, it is desirable that as many protein substances as possible be examined in regard to their cleavage products. While Mörner[1] has found that the protein substances, obtained from the lens of the ox, occupy a special position among the proteins, no detailed investigation of the cleavage products of the lens has been reported. Under these circumstances, it is worth while to report the following results of an investigation of the cleavage products of the crystalline lens of the ox as a contribution to the knowledge of the chemical composition of the lens tissue.

EXPERIMENTAL PART.

The material for the investigation was obtained from a nearby slaughter house and was always perfectly fresh. The lenses were freed from all foreign tissue and used immediately.

Water and Ash Content.

The water content of the fresh material was determined by drying to constant weight at about 105°C.

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[1] Mörner, C. T., *Z. physiol. Chem.*, 1894, xviii, 64.

Experiment No.	Fresh substance.	Substance lost.	
	gm.	gm.	per cent
1	2.2627	1.4352	63.46
2	2.2393	1.5113	64.06
3	2.1787	1.3872	63.67
4	2.3805	1.5546	65.36
5	2.3090	1.4832	64.23
6	2.6449	1.5929	64.04
7	2.6509	1.6294	65.25
Average...			64.30

Experiment No.	Fresh substance.	Ash.	
	gm.	gm.	per cent
1	2.3690	0.0187	0.79
2	2.4954	0.0192	0.77
3	2.5219	0.0184	0.73
Average...			0.76

Hydrolysis of the Lens by Hydrochloric Acid and Fractionation of the Esters of the Monoamino-Acids.

750 gm. of fresh lens were dissolved in 3 liters of concentrated hydrochloric acid by warming on the water bath, and the solution was boiled under a reflux condenser for 8 hours. A negative biuret reaction indicated that the hydrolysis was complete.

After separating 2.7 gm. of dark brown material by filtration, the filtrate was concentrated to a syrup under diminished pressure. The residue was taken up in 1 liter of absolute alcohol, the solution saturated with dry hydrogen chloride and boiled. The alcohol was then removed under diminished pressure and the esterification repeated three times. No glycocoll ester hydrochloride could be separated.

The esters in the residue were liberated by treatment with sodium hydroxide and extracted with ether in the presence of potassium carbonate. After drying 12 hours over sodium sulfate, the ether was distilled and the esters were fractionated.

Fraction.	Bath.	Temperature of Bath.	Pressure.	Amount.
		°C.	*mm.*	*gm.*
I	Water.	Up to 60	12	46.7
II	"	60–100	12	69.6
III	Oil.	100	0.5	4.0
IV	"	100–175	0.5	17.9

The residue weighed 97.2 gm.

Each of the first three fractions were boiled, under a reflux condenser, with 10 volumes of water until the reaction was no longer alkaline, and then evaporated to dryness under diminished pressure. In order to remove any proline the residues were boiled with absolute alcohol and the extract united. The proline was isolated in the usual manner as the copper salt. The active and racemic salts were separated by means of boiling absolute alcohol. The yields were 5.6 gm. of active, and 1.8 gm. of inactive copper salts, dried at 120°C.

0.1827 gm. of air-dry inactive copper salt lost 0.0196 gm. at 120°C.

	Calculated for $C_{10}H_{18}O_4N_2Cu.2H_2O$. per cent	Found. per cent
H_2O	10.99	10.37

0.1631 gm. of dry inactive copper salt gave 0.0437 gm. CuO.

	Calculated for $C_{10}H_{16}O_4N_2Cu$. per cent	Found. per cent
Cu	21.81	21.40

The free active proline melted at 211°C. An aqueous solution of the amino-acid was strongly levorotatory.

0.0992 gm. of substance neutralized 8.3 cc. of 0.1 N H_2SO_4.

	Calculated for $C_5H_9O_2N$. per cent	Found. per cent
N	12.17	11.72

Fraction I.—The material remaining after the extraction of the proline was suspended in absolute alcohol and dry hydrogen chloride introduced. No glycocoll was found. The solution was freed from hydrochloric acid by boiling with yellow lead oxide; the lead removed by treatment with hydrogen sulfide; and alanine and leucine were isolated as their copper salts, of which 3.2 and 0.2 gm., respectively, were found.

0.1138 gm. of alanine copper salt gave 0.0378 gm. of CuO.

	Calculated for $C_6H_{12}O_4N_2Cu$. per cent	Found. per cent
Cu......................................	26.50	26.56

The alanine hydrochloride was dextrorotatory.

0.1092 gm. of substance neutralized 11.8 cc. of 0.1 N H_2SO_4.

	Calculated for $C_3H_7O_2N$. per cent	Found. per cent
N......................................	15.73	15.38

Fractions II and III.—These fractions, after removal of the proline, were united and converted into copper salts. In order to separate the valine and isoleucine the mixture was exhaustively extracted with methyl alcohol according to Ehrlich and Wendel.[2] The soluble fraction was decomposed and the amino-acids were racemized by heating with aqueous barium hydroxide at 180°C. in an autoclave. The copper salts were again formed, and extracted with 96 per cent ethyl alcohol. On recrystallization of the soluble fraction, 0.2 gm. of copper salt was obtained. The analytical figures, unfortunately, did not check well with those of the copper salt of isoleucine. The yields of the copper salts of valine, leucine, and alanine were 3.2 gm., 17.2 gm., and 13.5 gm., respectively. There was a further yield of 3.7 gm. of free leucine.

Leucine.

0.1372 gm. of copper salt gave 0.033 gm. of CuO.

	Calculated for $C_{12}H_{24}O_4N_2Cu$. per cent	Found. per cent
Cu......................................	19.65	19.56

0.1653 gm. of leucine gave 14.6 cc. of moist nitrogen at 5°C. and 758 mm.

	Calculated for $C_6H_{13}O_2N$. per cent	Found. per cent
N......................................	10.69	10.77

The optical rotation of 0.6369 gm. was measured in 20 per cent hydrochloric acid. The total weight of the solution was 17.3998 gm. and its specific gravity 1.1. In a 2 dm. tube at 20°C. it rotated sodium light + 1.27°.

$$[\alpha]_D^{20} = + 15.77°$$

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[2] Ehrlich, F., and Wendel, A., *Biochem. Z.*, 1908, viii, 399.

Alanine.

0.1795 gm. of copper salt gave 0.0595 gm. of CuO.

	Calculated for $C_6H_{12}O_4N_2Cu$. per cent	Found. per cent
Cu.....................................	26.50	26.42

Valine.

0.2090 gm. of copper salt gave 0.0560 gm. of CuO.

	Calculated for $C_{10}H_{20}O_4N_2Cu$. per cent	Found. per cent
Cu.....................................	21.51	21.41

Fraction IV.—This fraction was worked up in the usual manner. After the phenylalanine ester had been extracted with ether, the insoluble residue was saponified by barium hydroxide and the glutamic acid separated as hydrochloride. The mother liquor was freed from hydrochloric acid and divided into two equal parts for independent estimations of the aspartic acid and serine. β-naphthaline sulfoserine could not be isolated in a perfectly pure state. 2.2 gm. of aspartic acid were recovered, equivalent to 3.5 gm. in the entire fraction. The yield of phenylalanine was 5.9 gm.

The phenylalanine, liberated by ammonia and crystallized from hot water, decomposed at 278°C. and gave the following analytical figures:

0.1158 gm. of substance neutralized 7.0 cc. of 0.1 N H_2SO_4.

	Calculated for $C_9H_{11}O_2N$. per cent	Found. per cent
N.....................................	8.49	8.47

The hydrochloride, which melted at 183°C., was also analyzed.

0.1023 gm. of substance contained 0.0177 gm. of chlorine.

	Calculated for $C_9H_{11}O_2N.HCl$. per cent	Found. per cent
Cl.....................................	17.59	17.31

The aspartic acid gave the following figures:

0.1079 gm. of substance gave 9.5 cc. of moist nitrogen at 5°C. and 763 mm.

	Calculated for $C_4H_7O_4N$. per cent	Found. per cent
N.....................................	10.53	10.76

Estimation of Tyrosine.

146 gm. of fresh lens were boiled with dilute sulfuric acid under a reflux condenser and the sulfuric acid was then quantitatively removed with barium hydroxide. The precipitated barium sulfate was repeatedly boiled with water until the Millon reaction was negative and the united filtrates were concentrated until crystallization began. After standing in the cold the tyrosine was filtered off and the concentration and crystallization were repeated until the filtrate was free from tyrosine. The fractions were then united and crystallized from hot water after treatment with boneblack. The yield of purified tyrosine was 2.3 gm. It melted at 293°C.

0.1141 gm. of substance neutralized 6 cc. of 0.1 N H_2SO_4.

	Calculated for $C_9H_{11}O_3N$. per cent	Found. per cent
N	7.74	7.37

A solution of the amino-acid in hydrochloric acid was levorotatory.

Estimation of Glutamic Acid.

The glutamic acid was isolated as hydrochloride. For this purpose, 323 gm. of fresh lens were hydrolyzed by boiling for 8 hours with 4 volumes of concentrated hydrochloric acid. The solution was then bone-blacked, concentrated under diminished pressure, and saturated with hydrogen chloride. After seeding it was allowed to stand at 0°C. When the crystallization was complete, the glutamic acid was filtered off, and the concentration and saturation repeated as long as a further crop separated.

The fractions were then united and, after treatment with animal charcoal, reprecipitated by hydrochloric acid, repeating, as before as long as more crystals could be obtained. The total yield of hydrochloride was 21.6 gm. It melted at 193°C. and gave the following figures on analysis:

0.1108 gm. of substance neutralized 5.8 cc. of 0.1 N H_2SO_4.
0.1121 gm. of substance contained 0.0215 gm. of chlorine.

	Calculated for $C_5H_9O_4N.HCl$. per cent	Found. per cent
N	7.63	7.35
Cl	19.31	19.18

Detection of Tryptophane.

90 gm. of fresh lens were incubated for 10 days in 300 cc. of water, saturated with chloroform, to which sodium carbonate and pancreatin (Merck) had been added. The tryptophane reaction was then positive.

Estimation of the Hexone and Purine Bases.

For this purpose, 1,025 gm. of fresh lens were heated under a reflux condenser on a Babo hot air funnel, according to Kossel and Kutscher,[3] with 3 parts by weight of sulfuric acid and 6 volumes of water until the Biuret test was negative. The solution was then freed from sulfuric acid by means of barium hydroxide and the filtrate and wash waters were united and concentrated. 5 per cent of sulfuric acid was then introduced and the hexone and purine bases were precipitated as tungstates. The tungstates were decomposed by barium hydroxide and the barium was removed as carbonate. The purine bases were precipitated from the resulting solution, and made weakly acid with dilute nitric acid by adding an excess of silver nitrate.

Purine Bases.—The silver precipitate was suspended in ammonium hydroxide and allowed to stand for 24 hours. The insoluble fraction was then decomposed by means of dilute hydrochloric acid, filtered, and evaporated to dryness. Only a very small amount of material, not enough for identification, was obtained when the residue, dissolved in water, was treated with an excess of ammonia.

The filtrate was freed from ammonia, weakly acidified with dilute hydrochloric acid, and treated with a saturated solution of sodium picrate. A crystalline precipitate was produced, which, after recrystallization from hot water, formed needles. The material melted at 279°C., the melting point of adenine picrate. The yield was too small for further identification.

The filtrate from the adenine picrate, after removal of the picric acid, gave no xanthine or hypoxanthine on treatment with ammoniacal silver solution.

Hexone Bases.—From the filtrate from the purine silver salts, the arginine and histidine were precipitated by silver nitrate and

[3] Kossel, A., and Kutscher, F., *Z. physiol. Chem.*, 1900, xxxi, 165.

barium hydroxide and filtered off. The histidine, after removing the silver and barium, was reprecipitated by mercuric chloride in the presence of carbonic acid.

The mercury was then removed and the histidine again passed through the silver-barium combination. After removal of the silver and barium, the solution was concentrated. One-fifth was converted into the picrolonate and the remainder into the hydrochloride. Owing to an accident the yield of picrolonate could not be determined. The hydrochloride recovered weighed 6.7 gm., indicating a total yield of 8.4 gm. It melted at 231° C. and gave the following analytical figures:

0.0945 gm. of substance contained 0.0291 gm. of chlorine.

	Calculated for $C_6H_9O_2N_3.HCl.$ per cent	Found. per cent
Cl......................................	31.14	30.79

The picrolonate blackened at 255° and melted at 265°C.

0.0652 gm. of substance gave 12.7 cc. of moist nitrogen at 12.5°C. and 759 mm.

	Calculated for $C_6H_9O_2N_3.(C_{10}H_8O_5N_4)_2.$ per cent	Found. per cent
N..	22.56	22.92

The filtrate from the histidine precipitate was completely freed from mercuric chloride by treatment with hydrogen sulfide and silver carbonate. The silver was then removed by hydrogen sulfide and the arginine precipitated as picrate. The yield was 27.5 gm. It melted at 205°C.

0.0712 gm. of substance gave 15.4 cc. of moist nitrogen at 22.5°C. and 760 mm.

	Calculated for $C_6H_{14}O_2N_4.C_6H_3O_7N_3.$ per cent	Found. per cent
N......................................	24.32	24.34

Silver and barium were removed from the filtrate from the histidine and arginine, and the solution was treated with phosphotungstic acid. The precipitates, after decomposition by barium

hydroxide and removal of the barium, were converted into hydrochlorides and the solution was evaporated to dryness. The choline in the residue was extracted with alcohol and precipitated from its alcoholic solution by mercuric chloride. The mercury compound was decomposed by treatment, in water, with hydrogen sulfide and the choline, after evaporation to dryness, dissolved in absolute alcohol, and precipitated with alcoholic chloroplatinic acid. Although an abundant flaky precipitate was formed, no perfectly pure choline chloroplatinate could be isolated.

One-fifth of the hydrochlorides, insoluble in alcohol, was converted into the picrate, and the mother liquor, after removal of picric acid, precipitated by phosphotungstic acid. The filtrate was then worked up for lysine picrate.

The lysine was also recovered from the alcoholic filtrate from the mercury salt of choline. The solution was treated with mercuric chloride and barium hydroxide, and the lysine, after removal of mercury and barium, isolated as picrate. The mother liquors were again worked up in the manner described above. The yield of lysine picrate was 5.8 gm. indicating 14.5 gm. were present. The picrate melted at 252°C. and gave the following figures on anaylsis:

0.1036 gm. of substance gave 17.2 cc. of moist nitrogen at 25.5°C. and 761 mm.

	Calculated for $C_6H_{14}O_2N_2.C_6H_3O_7N_3.$ per cent	Found. per cent
N	18.67	18.40

SUMMARY.

1. The hydrolysis of the lens of the ox yielded thirteen cleavage products: alanine, valine, leucine, aspartic acid, glutamic acid, lysine, arginine, phenylalanine, tyrosine, proline, tryptophane, histidine, and adenine.

2. Calculated to ash- and water-free substance the following quantities of amino-acids were found:

TABLE I.

Amino-acid.	Amount found.
	per cent
Alanine...	4.7
Valine...	1.0
Luecine...	6.8
Aspartic acid...	1.4
Glutamic acid...	15.5
Lysine......	1.6
Arginine...	3.3
Phenylalanine...	1.9
Tyrosine...	4.5
Proline...	2.2
Histidine...	1.6
Tryptophane..	Present.

In Table I, the relative quantity of glutamic acid is surprisingly high. This is due, in part to its direct isolation from a separate hydrolysis mixture.

3. The amounts of purine bases are surprisingly low.

DO THE AMINO-ACIDS OCCUR IN COW'S MILK?

By YOSHIZUMI HIJIKATA.

(From the Medico-Chemical Institute, Kyoto Imperial University, Kyoto.)

While it has been known for a long time that a small part of the total nitrogen contained in milk is in the form of non-protein substances, our knowledge of the various constituents, which make up this fraction, has been incomplete. The presence of urea, uric acid, hypoxanthine, guanine, adenine, etc.,[1] has been demonstrated, but whether the amino-acids occur as physiological constituents remains uncertain.

The leucine which Hoppe[2] found in milk which had been curdled for a considerable time may have arisen by hydrolysis. Recently, Denis and Minot[3] and these authors in collaboration with Talbot[1] found, with the aid of Van Slyke's micro apparatus, 4.03 to 4.5 mg. and 3.0 to 8.9 mg. of amino nitrogen in 100 cc. of cow's and human milk, respectively, indicating that normal milk may always contain small quantities of amino-acids. Owing to the limitations of the method, however, neither were the amino-acids characterized nor their quantities determined. Moreover, Van Slyke's method is by no means so specific as to enable an investigator to locate with certainty any traces of amino nitrogen in such a liquid. In view of these considerations, the presence of amino-acids in milk may not be considered proved until they have been isolated and characterized. This has been attempted and the following is a report of the investigation.

EXPERIMENTAL PART.

200 liters of fresh milk, from two cows, were freed from protein, in lots of 4 to 6 liters daily. Each lot was diluted with 2 volumes

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[1] Denis, W., Talbot, F. B., and Minot, A. S., *J. Biol. Chem.*, 1919, xxxix, 47, give a bibliography on this subject.

[2] Hoppe, F., *Arch. path. Anat. u. Physiol.*, 1859, xvii, 434.

[3] Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1919, xxxvii, 361.

of water and the casein precipitated by acidifying with dilute acetic acid.   Albumin was then removed by treatment with tannin solution, the excess of tannin precipitated by means of basic lead acetate and the excess of lead precipitated as sulfide.   The resulting protein-free solution was reduced to small volume and allowed to stand for a week with an equal volume of 60 per cent alcohol.

The filtrates from the crystallized lactose were combined, concentrated,[4] and sulfuric acid was added in sufficient quantity to make 5 per cent of the total volume.   The liquid was then filtered and the filtrate treated with phosphotungstic acid.   The precipitate, which formed, will be denoted as the phosphotungstic acid precipitate A and the filtrate as the filtrate from the phosphotungstic acid precipitate A.

Phosphotungstic Acid Precipitate A.

After trituration with 5 per cent sulfuric acid and washing, this precipitate was decomposed by barium hydroxide and the solution of the bases then treated in the manner described by Kossel and Kutscher.   The barium-free solution, weakly acidified with nitric acid, was precipitated by means of 20 per cent silver nitrate and filtered.   The precipitate was saved for identification of purine bases.

After adding silver nitrate to the filtrate until an excess was present, a cold saturated solution of barium hydroxide was added. This produced an abundant precipitate which was decomposed by means of sulfuric acid and hydrogen sulfide.   The filtrate was concentrated, 2.5 per cent of sulfuric acid introduced, and then a slight excess of mercuric chloride solution.   A yellow, flaky precipitate formed which, after standing over night, was filtered off and decomposed by means of hydrogen sulfide.   After removal of the hydrogen sulfide and sulfuric acid from the solution, since the diazobenzenesulfonic acid reaction was positive, alcoholic picrolonic acid was added.   After standing over night, the gelatinous precipitate was filtered off and repeatedly crystallized from hot water.   The material turned black at 225°C. and decomposed

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[4] A sample gave a definite ninhydrin reaction, while the biuret reaction was negative.

at 265°C. It gave analytical figures in accordance with those of histidine picrolonate:

0.0703 gm. of substance gave 14.2 cc. of moist nitrogen at 20°C. and 754 mm.

	Calculated for $C_6H_9O_2N_3.(C_{10}H_8O_5N_4)_2$. per cent	Found. per cent
N	22.56	22.82

The filtrate from the histidine mercuric sulfate was freed from mercury and sulfuric acid, and the concentrated solution treated with silver nitrate and barium hydroxide and filtered. After removal of the silver and barium, an excess of a saturated solution of picric acid was introduced. The silky, golden needles which precipitated melted, after recrystallization from hot water, at 201°C. and gave analytical figures for arginine picrate:

0.1040 gm. of substance gave 23.6 cc. of moist nitrogen at 27°C. and 758 mm.

	Calculated for $C_6H_{12}O_2N_4.C_6H_3O_7N_3$. per cent	Found. per cent
N	24.32	24.85

Silver and barium were removed from the filtrate from the first silver barium precipitate, the solution was concentrated at a low temperature and precipitated by phosphotungstic acid in the presence of 5 per cent of sulfuric acid. The precipitate was decomposed in the usual manner with barium hydroxide, the barium removed and, after acidifying with hydrochloric acid, the solution evaporated to dryness. The residue was exhaustively extracted with absolute alcohol and the united extracts were treated with alcoholic mercuric chloride. The further treatment of the precipitate will be recorded later.

The alcoholic filtrate from the above was treated with barium hydroxide and mercuric chloride. The material which separated was well washed and decomposed in water by sulfuric acid and hydrogen sulfide. Sulfuric acid was removed and the solution combined with the hydrochloride insoluble in alcohol. Phosphotungstic acid was then introduced in the presence of 5 per cent of sulfuric acid. The precipitate was decomposed in the usual manner and the base isolated as the picrate. The purified picrate melted at 252°C. and gave analytical figures which agreed with those of lysine picrate.

0.0724 gm. of substance gave 12.2 cc. of moist nitrogen at 22°C. and 751 mm.

	Calculated for $C_6H_{14}O_2N_2$ $C_6H_9O_7N_3$. per cent	Found. per cent
N..	18.67	18.76

Filtrate from the Phosphotungstic Acid Precipitate A.

The phosphotungstic and sulfuric acids were removed and the solution was concentrated to a syrup. A sample yielded a small quantity of β-naphthalin-sulfonate. The remainder, in the form of esters, was then fractionated. The amino-acids were esterified by dissolving them in absolute alcohol, passing in dry hydrogen chloride, and boiling. The process was repeated three times. The esters were liberated in the usual manner by sodium hydroxide and extracted with ether in the presence of potassium carbonate. The ethereal solution was dried over sodium sulfate, the ether distilled, and the residue, which was very small, fractionated.

Fraction.	Temperature.	Pressure.	Yield.
	°C.	mm.	gm.
I	Up to 60	12	1.3
II	60–100	0.5	0.5
III	100–180	0.5	1.1

Fractions I and II were separately hydrolyzed by boiling under reflux condensers with 10 volumes of water until the reactions were no longer alkaline and then evaporated to dryness. Proline was extracted from the residues by boiling with absolute alcohol. Some copper salt was isolated from the extract but not enough for analysis.

The alcohol-insoluble part of Fraction I was examined for glycocoll by esterification and crystallization of the ester hydrochloride but without success. The ester was hydrolyzed, alcohol and hydrochloric acid were removed and, after uniting with that part of Fraction II insoluble in absolute alcohol, was boiled with an excess of copper oxide. In this way crystalline copper salts were obtained but not in sufficient quantity for analysis.

Fraction III was extracted with ether in order to separate any phenylalanine ester, saponified, and examined for glutamic acid. It was not possible, however, to isolate quantities of crystalline substance that would suffice for analysis.

Purine Base Fraction.

The precipitated silver salts (page 166) were heated in dilute ammonia and filtered. The insoluble fraction was then decomposed by heating with dilute hydrochloric acid, the solution filtered and evaporated to dryness. The residue, dissolved in water, gave a brownish precipitate with an excess of ammonia. The precipitate was purified by solution in sodium hydroxide and reprecipitated with acetic acid. A part of the material was then dissolved in dilute sulfuric acid and the solution allowed to evaporate slowly. Long macroscopic needles formed such as have been described for guanine sulfate. The remainder of the acetic acid precipitate was converted into the picrate, which crystallized in bundles of fine orange-colored needles. When heated to nearly 170°C. the picrate became clearer and decomposed gradually without melting. Although the amount of material isolated was too small for analysis, the properties recorded seem sufficient to establish the presence of guanine.

Ammonia was expelled from the filtrate from the guanine silver salt by heating on the water bath and the solution, acidified with hydrochloric acid, treated with a saturated sodium picrate solution. The amorphous precipitate, which formed, was recrystallized from hot water. It then melted at 282°C. and gave analytical figures corresponding to adenine picrate.

0.0583 gm. of substance gave 14.6 cc. of moist nitrogen at 205°C. and 752 mm.

	Calculated for $C_5H_5N_5.C_6H_3O_7N_3.$ per cent	Found. per cent
N	30.78	30.49

Picric acid was removed from the filtrate from the adenine picrate and the solution treated with silver nitrate in the presence of nitric acid. The precipitate was decomposed with hydrochloric acid and evaporated to dryness. The evaporation was repeated several times with alcohol and water to completely remove the

excess of hydrochloric acid. The residue digested in water at 40°C. gave a very slight precipitate. The Weidel reaction was negative. The filtered solution gave a precipitate with dilute picric acid solution only after concentrating and cooling. The picrate formed macroscopic tabular crystals which melted at 210°C. Unfortunately its identity with hypoxanthine picrate could not be established with certainty.

Lysine Fraction.

The mercury was removed from the alcoholic solution (page 167) and the solution evaporated to dryness. The filtered alcoholic solution of the residue gave a precipitate with alcoholic chloroplatinic acid. The precipitate was washed with alcohol, dissolved in hot water, and the solution allowed to evaporate. The chloroplatinic acid separated in well formed orange-colored prisms which melted at 234°C. and gave analytical figures for choline chloroplatinate:

0.1806 gm. of substance gave 7.8 cc. of moist nitrogen at 22°C. and 749 mm.

0.1446 gm. of substance gave 0.0453 gm. of platinum.

	Calculated for $(C_5H_{14}ONCl)_2PtCl_4.$ per cent	Found. per cent
N.	4.54	4.80
Pt.	31.65	31.33

SUMMARY.

1. The following amino-acids have been found in cow's milk: lysine, arginine, and histidine.

2. Monoamino-acids, also, probably are present.   Further investigation is necessary, involving a larger quantity of material.

ADDENDUM.

In addition to the amino-acids enumerated above, the following substances were found: guanine and adenine in the purine base fraction (page 166); and choline in the lysine fraction (page 167).

THE KIDNEY FACTOR IN PHLORHIZIN DIABETES.

By THOMAS P. NASH, Jr.

(*From the Department of Chemistry, Cornell University Medical College, New York City.*)

It has been repeatedly emphasized that the distinctive symptom of phlorhizin diabetes is the subnormal concentration of sugar in the blood. Von Mering (1) was the first to recognize this characteristic hypoglycemia, and to suggest, in explanation thereof, an increased permeability of the kidney to blood sugar.

Opposed to the theory of von Mering is the view of Levene (2) and others that the kidneys in a phlorhizinized animal do not serve as a simple filter, but actively produce sugar. Levene compared the sugar in the arterial and venous blood of the kidneys of phlorhizinized dogs. Of nine dogs thus examined, eight showed a higher concentration of sugar in the renal vein than in the renal artery, the maximum difference observed being 0.024 per cent and the average difference (in the eight positive cases) being approximately 0.010 per cent.

Zuntz (3) criticized Levene's results on the ground that the experimental procedure employed (the introduction of a cannula into the renal vein) blocked, at least temporarily, the renal circulation. According to Zuntz, even a temporary block in the renal circulation stops urine secretion for a considerable time, during which period no decrease in the sugar content of the renal venous blood could be expected. The actual increases found by Levene are of such an order, according to Zuntz, as to fall within the limits of experimental error.

Levene's work, nevertheless, has received substantial corroboration. Biedl and Kolisch (4), although they cite no experimental data, state that in phlorhizinized animals the blood of the renal vein contains more glucose than does arterial blood, particularly when the urine secretion is restricted; with profuse urine secretion

the glucose content of renal venous blood is frequently the same as of arterial blood, although even here cases are found in which the renal vein shows significantly more sugar than the artery. Further, these authors find after giving phlorhizin, whether to dogs or rabbits, a hyperglycemia. Pavy, Brodie, and Siau (5) found, after ablation of the abdominal viscera (except kidneys), thus removing the presumed mechanism for reinstating the blood sugar necessitated by the theory of von Mering, that phlorhizin injection produces the usual glycuresis. They also confirm Zuntz (3), that injection of phlorhizin into the renal artery of one kidney produces glycuresis from that kidney before the other kidney is involved. But where Zuntz interpreted his results to mean that phlorhizin alters the normal "Anziehungs-kraft" of the renal epithelium for sugar, Pavy, Brodie, and Siau take the view that

".   .   .   .   the glycosuric effect of phloridzin is attributable to a specific action exerted upon the cells of the renal tubules by which they acquire the power of producing sugar   .   .   .   .   under the influence of the presence of phloridzin these cells exert a katabolising action upon something reaching them from the blood, resulting in the liberation of dextrose in a manner comparable to that by which lactose is set free by the cells of the mammary gland."

Lépine and Boulud (6) observed "frequently" in the blood of the renal vein (collected according to the method of Biedl and Kolisch (4), so as not to disturb the renal circulation) more "immédiat" sugar than in the carotid artery. In one case where the carotid blood showed 0.44 part of immediate sugar per 1,000 parts of blood, the renal venous blood showed 1.06 parts. Again, 6 years later, Lépine (7), answering Erlandsen's (8) contention that the essential cause of phlorhizin glycuresis is a temporary augmentation of "l'aptitude du rein" to excrete sugar, insists that it is absolutely indisputable that more sugar is often found in the renal venous blood of phlorhizinized animals than in the arterial blood.

Coolen (9) found in phlorhizinized rabbits a hyperglycemia which was augmented by extirpation of the kidneys. He came to the conclusion that phlorhizin glycuresis could not be attributed to a renal origin, but is probably responsive to the same mechanism as all other known glycureses.

Underhill (10) found in completely phlorhizinized dogs, after ligation of the renal structures, and in phlorhizinized rabbits whose kidney function had been abolished through subcutaneous administration of tartrate, a significant hyperglycemia. He views this evidence as corroborative of von Mering's theory and indicative also of an action exerted by phlorhizin upon other structures than the kidney resulting in the increased production of sugar.

There is no longer any question that phlorhizin glycuresis is accompanied by a definite and characteristic hypoglycemia. Moreover, the discovery by Lusk and coworkers (11) that glucose fed to the completely phlorhizinized animal is recovered quantitatively in the urine as "extra sugar" has been taken to support the von Mering theory, since the failure of the organism to burn sugar may be due not necessarily to any impairment of the mechanism for combustion but to so rapid elimination of the sugar from the blood as to keep the concentration below a minimal value required for the operation of this mechanism. Hence, despite the conflicting views cited above, it is now generally accepted that phlorhizin glycuresis is due, at least directly, to a lowered renal threshold for blood sugar. It may be pointed out, however, that the fact of hypoglycemia and the hypothesis of increased renal permeability are not of necessity irreconcilable with an active production of sugar by the kidney. It is entirely conceivable that the kidney might develop both of these capacities simultaneously and interdependently. A definitely increased concentration of sugar in the renal venous blood would be difficult to account for on any basis other than that of sugar production in the kidney; and it does not seem justifiable to disregard the analyses pointing to this possibility merely on the assumption of error in the gross technique employed. So far as we know these analyses have never been experimentally refuted, and we have found no analyses showing the opposite result; *viz.*, a loss of sugar in blood which has traversed the renal circulation. In order to dispose conclusively of the possibility that the phlorhizinized kidney produces, at least in part, the sugar which it excretes, it would appear to be essential to demonstrate not only a loss of blood sugar in passing the kidney, but a loss of such an order of magnitude as to account substantially for the excreted sugar. The question has seemed to us of sufficient interest to

justify its reinvestigation, employing one of the more accurate techniques for blood sugar estimation which has the further advantage of requiring only a small quantity of blood.

GENERAL PROCEDURE.

Our experimental subjects have been, uniformly, female dogs. These animals received daily throughout the experimental period subcutaneous injections of 1 gm. of phlorhizin rubbed up in sterile olive oil. The experimental period in six of the eight cases was extended over several days in order to demonstrate the degree of glycuresis and observe the development of the attendant hypoglycemia. The later animals of the series were fed meat in order to increase the total sugar output and thus, presumably, the difference, if any, between the sugar content of arterial and renal venous blood.

A point signally overlooked by Levene, and of which only a generalization is made by Biedl and Kolisch, is the relation between the sugar content of the renal venous blood and the secretory activity of the kidney at the time blood is taken for analysis. This consideration is of prime importance since it is generally recognized that anesthesia impairs renal function to an unpredictable degree (12). In terms of the von Mering theory, if the kidney secretion has been seriously checked we should expect to find little if any difference in the sugar of the blood after its passage through the kidney; whereas, if the kidney is producing sugar, we should expect a much higher concentration of sugar in the renal vein when the urine secretion is diminished or suspended. In our experiments, therefore, we have taken the arterial and renal venous bloods only after a 2 hour period of ether anesthesia, during which time the urine was collected, its volume noted, and its sugar content determined.

In several cases, shortly before anesthesia, water was given by stomach tube to the animals, with a view to counteracting the tendency of the anesthetic to diminish urine secretion. Having regard to the effect of hemorrhage in increasing the sugar content of the blood, the amounts of blood taken have in all cases been limited to the small quantities (in few instances more than 5 cc.) required for analysis. Furthermore, the comparative blood samples, following 2 hours of anesthesia, have been taken as

nearly simultaneously as practicable, the technique employed being: Near the end of the anesthesia period the femoral or carotid artery was exposed, and a glass cannula inserted. Timing the operation, a midline abdominal incision was next made, the renal vein on one side exposed, and a loose ligature passed under the vein close to the kidney.. A curved needle attached by a piece of rubber tubing to a pipette containing a pinch of potassium oxalate·was inserted into the renal vein, the point of the needle towards and close to the kidney, and about 5 cc. of blood were quickly drawn into the pipette. The ligature was now tied to prevent subsequent hemorrhage, the needle was withdrawn, and at once a small quantity of arterial blood·was taken from the cannula directly into a test-tube containing potassium oxalate.[1] It is to be noted that the renal blood was thus taken without disturbing the normal circulation through the kidney.

In all samples of blood collected potassium oxalate was used to prevent clotting. Blood sugar was measured by the modified method of Lewis and Benedict (13); urinary sugar by the Allihn method, weighing the copper as cupric oxide; total nitrogen by the macro Kjeldahl technique. Merck's phlorhizin was used.

EXPERIMENTAL.

Dogs 24 and 25.—These dogs, in the course of another experiment, had received daily injections of 1 gm. of phlorhizin in olive oil, and were available for use in the work here reported only when they had reached a late stage of phlorhizin glycuresis. Further preliminary observation was not practicable, and the animals were accordingly submitted at once to ether anesthesia, which marks the final stage of the experimental routine as outlined above. The experimental data obtained are shown in Table I.

Dogs 26, 27, 29, 30, 31, and 32.—The data covering the whole experimental period for each of these dogs are given in Table I.

The 24 hour urines were marked off by catheterization and irrigation of the bladder at 10.00 o'clock in the morning. Except as otherwise indicated in Table I, blood was next taken from the

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[1] The author wishes to express to Mr. Cecil Dudley appreciation for assistance in the operations.

jugular vein by a needle inserted through the skin.   The phlorhizin injection, and feeding (where food was given at all), were completed by 10.30.

TABLE I.

Data of the whole experimental period.   Subcutaneous injections of 1 gm. of phlorhizin in olive oil, daily at 10.30 a.m.

Date.	Urine.				Blood sugar			Remarks.
	24 hr. volume.	Total nitrogen.	Total sugar.	D:N	Jugular vein.	Femoral artery.	Renal vein.	
Dog 24.								
	cc.	*gm.*	*gm.*		*per cent*	*per cent*	*per cent*	
June 6	13*	0.12	0.75	6.25		0.25†	0.25	After 2 hrs. ether anesthesia.
Dog 25.								
June 16					0.08			Blood taken just before anesthesia.
June 16	42*	0.39	2.87	7.36		0.14†	0.12	Blood taken after 2 hrs. ether anesthesia.
Dog 26. Weight, 8.86 kilos.								
June 13					0.13			
" 14	570	5.47	12.90	2.35	0.11			Three injections of adrenalin (0.04 mg. per kilo) at 3 hour intervals.
" 15	625‡	5.94‡	16.06‡	2.70	0.11			Four injections of adrenalin, as above.
" 16	710	7.31	15.80	2.16	0.09			
" 17	565	6.83	11.94	1.75	0.09			Etherized, 10 to 12 a.m.; 4 to 5 p.m.
" 18	280‡	4.57‡	8.52‡	1.86		0.10†		Carotid blood taken just before anesthesia.
" 18	2*					0.12†	0.12	After 2 hrs. ether anesthesia.

* Volume of urine secreted during the 2 hour anesthesia period.
† Carotid artery.
‡ 24 hour volume incomplete.

TABLE I—*Continued.*

Date.	Urine.				Blood sugar.			Remarks.
	24 hr. volume.	Total nitrogen.	Total sugar.	D:N	Jugular vein.	Femoral artery.	Renal vein.	
	cc.	*gm.*	*gm.*		*per cent*	*per cent*	*per cent*	

Dog 27.   Weight, 6.18 kilos.

Date.	24 hr. volume.	Total nitrogen.	Total sugar.	D:N	Jugular vein.	Femoral artery.	Renal vein.	Remarks.
June 28					0.14			
" 29	195	2.35	13.54	5.76				Fed 61 gm. lean, cooked meat.
" 30	188	5.73	17.24	3.01	0.10			Fed 100 gm. lean, cooked meat.
" 30	34*	0.47	3.84	8.17		0.21	0.17	100 cc. water by stomach tube at 1 p.m. Blood taken after anesthesia from 2 to 4 p.m.

Dog 29.   Weight, 9.70 kilos.

Date.	24 hr. volume.	Total nitrogen.	Total sugar.	D:N	Jugular vein.	Femoral artery.	Renal vein.	Remarks.
Oct. 5					0.13			
" 6	550	9.40	36.24	3.85				Fed 100 gm. lean, cooked meat.
" 7	625	14.48	41.68	2.87	0.09			" " "
" 8	555	13.43	39.52	2.94	0.08			" " "
" 8	16*	0.23	0.86	3.74		0.26	0.26	100 cc. water by stomach tube at 1 p.m. Blood taken after ether anesthesia from 1.50 to 3.50 p.m.

Dog 30.   Weight, 6.80 kilos.

Date.	24 hr. volume.	Total nitrogen.	Total sugar.	D:N	Jugular vein.	Femoral artery.	Renal vein.	Remarks.
Oct. 10					0.13			Fed 100 gm. lean, cooked meat.
" 11	240	6.95	24.28	3.49				Fed 50 gm. lean, cooked meat (all animal would eat).
" 12	320	8.79	29.35	3.34	0.08			" " "
" 13	310	8.78	28.24	3.21				" " "
" 14	300	7.08	20.64	2.91		0.08		Animal refused meat.
" 14	34*	0.39	1.77	4.54		0.08	0.07	400 cc. water by stomach tube at 1 p.m. Blood taken after ether anesthesia from 2.40 to 4.40 p.m.

TABLE I—*Concluded.*

Date.	Urine.				Blood sugar.			Remarks.
	24 hr. volume.	Total nitrogen.	Total sugar.	D.N	Jugular vein.	Femoral artery.	Renal vein.	
	cc.	*gm.*	*gm.*		*per cent*	*per cent*	*per cent*	
Dog 31. Weight, 8.78 kilos.								
Oct. 24					0.13			Fed 150 gm. lean, cooked meat.
" 25	815	8.78	27.00	3.07				" " "
" 26	940	10.45	30.19	2.89	0.10			" " "
" 27	850	9.84	30.48	3.09				Fed 110 gm. lean, cooked meat (all animal would eat).
" 28	810	8.37	27.44	3.27	0.09			Fed .150 gm. lean, cooked meat.
" 28	34*	0.43	3.30	7.67		0.13	0.12	500 cc. water by stomach tube at 1.25 p.m. Blood taken after ether anesthesia from 2.45 to 4.45 p.m.
Dog 32. Weight, 10.02 kilos.								
Oct. 31					0.11			Fed 150 gm. lean, cooked meat.
Nov. 1	335	8.14	30.74	3.77				Fed 65 gm. lean, cooked meat (all animal would eat).
" 2	455	10.00	32.96	3.29	0.07			Fed 150 gm. lean, cooked meat.
" 3	640	12.96	41.64	3.21				" " "
" 4	785	12.23	40.46	3.31	0.10			" " "
" 4	60*	0.79	6.24	7.90		0.17	0.14	300 cc. water by stomach tube at 1.05 p.m. Blood taken after ether anesthesia from 2.30 to 4.30 p.m.

Dog 26, as shown in Table I, received on the 2nd and 3rd days of the experimental period subcutaneous injections of adrenalin (14). Again, on the day preceding the conclusion of the experi-

ment, the dog was subjected to two periods of anesthesia. This regimen was designed to deglycogenize the animal and prevent hyperglycemia during the final anesthesia, but led to such impairment of renal function that it was not employed in the later dogs. On 2 days this dog voided outside of the cage; hence the 24 hour urines corresponding to these days were incomplete.

The last five dogs of the series received warm water by stomach tube a short time before they were anesthetized.

With Dogs 31 and 32 the urine secreted during the 2 hour period of anesthesia was measured at 30 minute intervals, in order to determine whether the effect of the anesthetic was constant or progressive. The following results were found:

	Dog 31.	Dog 32.
	cc.	*cc.*
First 30 minutes.............................	11	13
Second 30 "	6	14
Third 30 "	8	18
Fourth 30 "	9	15

DISCUSSION.

Inspection of Table I reveals in the last six cases a typical picture of phlorhizin glycuresis, accompanied by a definite hypoglycemia. In several instances following the injection of phlorhizin the blood sugar has fallen until it is little more than one-half the normal value.

Following ether anesthesia we have observed in every case except Dog 30 an increase in blood sugar. This observation is in general accord with that of other workers (15). The increase in the case of Dog 29 was more than 200 per cent above the value just prior to anesthesia, and in general was above the normal value. The degree of hyperglycemia is determined both by kidney activity and the apparent glycogen reserve of the animal. Thus Dog 26, which had received a series of injections of adrenalin during the fore period, secreted only 2 cc. of urine under anesthesia, yet the percentage of blood sugar increased only from 0.10 to 0.12. Dog 27 secreted 34 cc. of urine containing 3.8 gm. of sugar during the ether period, yet the blood sugar increased

from 0.10 to 0.21 per cent; in this case the high D:N ratio of 8.17 is indicative of an unusually high glycogen reserve. In striking contrast is Dog 30, which also secreted 34 cc. of urine during anesthesia, but only 1.7 gm. of sugar. Here the blood sugar showed no change, and the relatively low D:N ratio of 4.54 bears out the probability of a depleted glycogen reserve prior to anesthesia. With regard to this point we find in our notes, under date of October 12, a memorandum that the weather had suddenly turned cold, and the dog, lying in a metal cage in an unheated room, was constantly shivering. It is generally accepted that shivering will deglycogenize a phlorhizinized dog.

A comparison of the sugar concentration in arterial and renal venous blood shows that in no case have we found more sugar in the renal blood. On the contrary, in five of the eight dogs we have found appreciable differences in favor of the arterial blood sugar. It will be noted, furthermore, that in the three cases where we found no change in the blood sugar content the amounts of sugar excreted during the anesthesia period were the smallest obtained in any of the experiments (less than 1 gm.). The greatest observed loss in blood sugar in passing through the kidney was 0.04 per cent, Dog 27, and in this case the sugar excreted during the 2 hours was 3.84 gm. The average excess of sugar in the arterial blood of the five positive cases was 0.022 per cent.

It is significant that there is a direct proportion between the sugar excreted in the urine and the loss from the blood. Whether the loss from the blood is sufficient to account for the sugar recovered in the urine is a question which obviously involves other and uncertain factors. Not only must the average volume flow of blood through the kidney be known, but it must be assumed that this average rate obtained at the moment of drawing the renal blood. If the rate of blood flow through the kidneys is 150 cc. per minute per 100 gm. of kidney tissue (16), and the average weight of kidney tissue in the dogs of our experiment was 60 gm., the average volume of blood passing through the two kidneys during the anesthesia period was 10,800 cc. With an average loss in arterial blood sugar of 0.022 per cent, we could thus account for 2.38 gm. of excreted sugar. The total sugar excreted during the anesthesia period by the five dogs upon which

the calculation is based was 18.02 gm., an average of 3.6 gm. per dog. In other words the amount calculated is 66 per cent of the amount found. In the case of Dog 27, the actual sugar excreted was 3.84 gm., as compared with a calculated value of 4.32 gm. We believe, in view of the several indeterminate factors involved, that the correspondence between actual and calculated excreted sugar is close enough to warrant the conclusion that the phlorhizinized kidney is not concerned in a function of specific sugar production.

CONCLUSION.

In phlorhizinized dogs the renal venous blood shows a lower concentration of sugar than the general arterial blood, provided the kidney function has not been abolished or seriously impaired. Failure to control the effect upon the kidney of general anesthetics, and inherent errors in the methods employed for blood collection and sugar estimation, account for contrary results previously reported.

It appears that the kidneys, under the influence of phlorhizin, do not acquire a specific sugar-producing function. Our results confirm an increased permeability of the renal epithelium.

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NOTE ON THE AMMONIA CONTENT OF BLOOD.

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In a personal communication, Dr. T. Addis, of Stanford University Medical School, has suggested that the increased ammonia which we find (1) in blood of the renal vein may be due to return of previously excreted ammonia from a kidney which has ceased to function normally under the experimental conditions necessary in drawing the blood. Addis and Shevky (2) have reported an increase of urea in renal venous blood of rabbits at a time when no urine was being secreted.

The suggestion made by Dr. Addis seemed to us of sufficient interest to warrant a presentation of some considerations which we believe conclusively show that the increased ammonia content of the blood of the renal vein cannot be explained upon such a basis.

Addis and Shevky found more urea in the blood of the renal vein only when urine secretion had ceased after clamping and ligating the renal vessels of the opposite side. When blood was taken quickly the renal venous blood showed, usually, a loss of urea. In our animals, except those whose urine secretion was controlled over a definite period (see below), the renal blood was taken as quickly as anesthesia could be effected and the operation performed; the kidney on only one side was exposed, and this disturbed as little as possible; and blood was taken from the vein without even a temporary stoppage of the renal circulation. We have found, *without exception*, more ammonia in the renal vein than in other blood, and we obtained this finding regardless of whether the blood was taken early or late in anesthesia. Such results are not to be expected if one is dealing with an effect of the

anesthesia upon urine secretion. That we were not dealing with such an effect is definitely proved by the following considerations:

In those animals from which blood was taken late in anesthesia the urine secretion was controlled. Thus, we reported a dog which received intravenously during 2 hours of ether anesthesia 196 cc. of 2 per cent sodium bicarbonate solution. At the end of this period (during which a total of 81 cc. of urine was taken by catheterization at 30 minute intervals), the renal venous blood now contained 0.18 mg., and the femoral arterial blood 0.07 mg., of ammonia nitrogen per 100 cc.

Dogs 24, 25, and 27 were diabetic from phlorhizin injection. From each of these dogs blood was taken after a 2 hour ether anesthesia during which period the urine was collected by catheter,

TABLE I.

Dog No.	Urine during 2 hrs. ether anesthesia.			Blood at end of 2 hrs. ether anesthesia.			
				Carotid.		Renal vein	
	Volume.	Total N.	Total sugar.	NH₃-N per 100 cc.	Sugar.	NH₃-N per 100 cc.	Sugar.
	cc.	gm.	gm.	mg.	per cent	mg.	per cent
24	13	0.12	0 75	0.14	0.25	0.25	0 25
25	42	0.39	2.87	0.13	0.14	0.18	0.12
27	34	0.47	3.84	0.05	0.21	0.14	0.17

its volume noted, and its sugar content determined. The same sample of each blood was analyzed for ammonia and sugar. The blood sugar values are included in a series recently reported (3); they were not given in our original paper dealing with the ammonia content of the blood because they were intended as a separate investigation, and in only the three dogs did our problems overlap. The comparative data for these dogs are summarized in Table I.

It will be seen that Dog 24 shows no change in the sugar concentration of the renal blood, but a marked increase in the ammonia content. The other two dogs (which were secreting urine more freely) show a definite decrease in the sugar content of the blood after traversing the kidney, while the ammonia content of the same blood sample is increased (in one case to

nearly 300 per cent of its former value). These facts further substantiate our conclusion that ammonia is formed by the kidney.

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THE DETERMINATION OF URIC ACID IN BLOOD.

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The determination of uric acid has been usually regarded as the least accurate and the most tedious and exacting of any of the analyses commonly made upon the blood. Certainly the heat coagulation method followed by concentration and precipitation with silver magnesia mixture as proposed by Folin and Denis (1) and modified by the present writer (2) was a laborious process, and required relatively large amounts of blood and careful analytical work for satisfactory results. Folin and Wu (3) have recently suggested a very interesting procedure which provides for direct precipitation of uric acid from highly diluted blood filtrates. After decomposing the precipitate with sodium chloride in hydrochloric acid solution the uric acid is determined practically as formerly, but instead of using 10 to 20 cc. of blood for a determination, as in the older process, Folin and Wu made use of an equivalent of only 2 cc. of blood for the development of color in their method. The depth of color thus obtained in most bloods is exceedingly weak. To the mind of the present writer it is questionable whether the great advance made by Folin and Wu in precipitating the uric acid directly from the dilute blood filtrate is not more than offset by having the final solutions so weak in color that it is questionable whether they can be compared accurately in a colorimeter by most analysts.

The observation of the writer several years ago that cyanide caused an increase of the color given by uric acid and phosphotungstic acid in alkaline solution offered the possibility of greatly increasing the color obtainable from a given quantity of uric acid. With a large excess of cyanide (several cc. of a 5 per cent solution of potassium or sodium cyanide) the reaction was found to become

exceedingly delicate, but advantage could not be taken of this fact for analytical purposes for two reasons. With the large excess of cyanide the color reaction was found not to be closely enough proportional to the quantity of uric acid present. Furthermore, turbidity always developed in such solutions, which was a very objectionable feature in analytical work, as the precipitate would continue to form slowly over a considerable period of time. The results obtained in this connection did, however, lead us to keep in mind the possibility of developing a more satisfactory technique for uric acid determination, based upon the use of large quantities of cyanide. It seemed possible that the reaction might be thus made more specific for uric acid, so that the determination could be made directly upon the blood filtrate, without preliminary precipitation of the uric acid. Experiments along this line have been carried on from time to time for the past few years, but the results obtained have hitherto been unsatisfactory for one reason or another. Last September experiments were begun in which the possible application of arsenic tungstic acid in uric acid determination was studied. Results obtained in this connection have proved very satisfactory, and the purpose of the present paper is to describe new uric acid reagents based upon the use of arsenic and phosphoarsenic tungstic acids, and to present a method for uric acid determination in blood which can be applied directly to small quantities of the Folin-Wu blood filtrate.

Arsenic tungstic acids have been described by Kehrmann (4), Fremery (5), and Gibbs (6). In the present work it was soon found that boiling sodium tungstate and arsenic acid together in solution would give a reagent which could be used for uric acid determination, but the condensation of the two acids under such conditions was very slow. Long boiling was required, and the solutions were apt to become quite strongly colored during the continued heating. We, therefore, added hydrochloric acid as a condensing agent. Using this acid, it was found that arsenic and tungstic acids will react very promptly in hot solution, so that the reaction is complete within 20 minutes or less, and the solution colors even less than do the phosphotungstic acids. Our very first experiments showed that reagents prepared in this way are definitely superior to the older phosphotungstic acid reagents, even when used in exactly the same way. Under such

conditions they develop about 20 per cent more color than do the phosphotungstic acid reagents, and are very much less apt to yield turbidity. It was found using 10 gm. of sodium tungstate, and condensing the tungstic acid derived from this by means of hydrochloric acid with 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 gm. of arsenic pentoxide and making to a final volume of 100 cc., that any quantity of the arsenic pentoxide above about 1.8 gm., yields a reagent with a color-yielding power equal to the solution obtained when any of the larger quantities of arsenic acid are used.

We shall describe the arsenic tungstic acid reagent which we first employed, though we no longer use it. It may be of service to those who wish to continue to use the old precipitation method for uric acid determination, since it is more easily prepared than is the old phosphoric acid reagent, and is remarkably free from any tendency to formation of turbidity. This reagent is prepared as follows: 100 gm. of sodium tungstate (J. T. Baker's c. p. is satisfactory, or the "Primos" brand may be used if obtainable) and 30 gm. of pure arsenic acid (As_2O_5) are placed in a liter flask and about 700 cc. of water added. 50 cc. of concentrated hydrochloric acid are then added and the mixture is boiled for about 20 minutes. It is then cooled and diluted to 1 liter.

This reagent may be employed exactly as is the phosphotungstic acid reagent of Folin and Denis, and is to be recommended in place of that reagent to anyone who may prefer to continue using the old method. We found, however, that with the new arsenic tungstic acid reagent it is possible to use an excess of sodium cyanide solution, and to dispense with the use of sodium carbonate. Under such conditions a much deeper color develops for a given quantity of uric acid than can be obtained under the old condition.

The color obtainable with the new reagent under the new condition was so great that it seemed probable that the new reaction would be much more specific for uric acid than was the old. Hence we were led to try the reaction directly upon blood filtrates and also compared the relative color given by a typical "interfering substance" in the new process. The results obtained here fell just short of our hopes. Resorcinol, used in minute quantities, would give about one-fourth to one-third as much color as

an equal weight of uric acid,[1] and when the reaction was applied directly to blood filtrates, a considerable proportion of bloods yielded distinctly high figures by the direct method, while others gave figures which agreed closely with those obtained by the precipitation method of Folin and Wu employed as described later in this paper.

As the results obtained in this connection so nearly fulfilled our expectations we were led to investigate the possibilities further before abandoning the hope of obtaining a reagent which could be applied directly to blood filtrates.

We therefore tried, among other things, combining tungstic acid with both arsenic and phosphoric acids. Curiously enough a reagent was thus obtained which is certainly more specific for uric acid than either arsenic tungstic or phosphoric tungstic acid alone, and as we shall see below, we believe that the new reagent fulfills reasonable demands for direct determination of uric acid on the Folin-Wu filtrates from human blood.

The reagent used in the method described below is prepared as follows: 100 gm. of pure sodium tungstate are placed in a liter flask and dissolved in about 600 cc. of water. 50 gm. of pure arsenic pentoxide are now added, followed by 25 cc. of 85 per cent phosphoric acid and 20 cc. of concentrated hydrochloric acid. The mixture is boiled for 20 minutes, cooled, and diluted to 1 liter. The reagent appears to keep indefinitely.

When used as described below this arsenic phosphotungstic acid reagent yields nearly seven times as much color from a given weight of uric acid as does the old phosphoric acid reagent as formerly employed. The new reagent is scarcely affected by a typical polyphenol such as resorcinol in the presence of uric acid. If five times as much resorcinol as uric acid be present in a solution, the quantity of color obtained is increased by only about 15 per cent, and there is no influence on the shade or quality of color produced. With fifty times as much resorcinol as uric acid, there is about 66 per cent increase in the color obtained. It is

[1] It is not correct to generalize regarding the amount of color given by resorcinol and uric acid with the old (or new) reagent, since under the conditions employed for uric acid determinations the color given by resorcinol is only very roughly proportional to the quantity of the substance present.

interesting to note that if no uric acid be present, resorcinol yields considerably more color than when even very minute amounts of uric acid are contained in the solution. This is probably best explained by assuming that the uric acid reacts first with the color-yielding substance in the reagent.

Since the substances in blood, other than uric acid which give color with the old reagent are unknown (though it is commonly assumed' that they are probably polyphenols), it is obvious that the true specificity of a reagent for uric acid determination in blood can be determined only by comparing the results obtained when the reaction is applied directly to blood filtrates with those obtained by the use of some accepted precipitation method. We have made such comparison in some detail for human blood, and the findings will be discussed below.

The technique of the new method for uric acid determination in human blood is as follows.

Standard Solutions.—The color obtained in the new method from a given quantity of uric acid is so intense that the standard solutions employed have a concentration of uric acid considerably below the solubility of uric acid in pure water. For this reason we are able to employ as standard, solutions of uric acid which are strongly acid with hydrochloric acid. We shall report later upon the keeping quality of these solutions. At present we prepare them fresh once in 2 weeks by appropriate dilution of the phosphate standard solution described by Benedict and Hitchcock in a previous paper. Unless kept in an excessively warm room, the phosphate standard may be relied upon to keep about 2 months.

It is desirable to keep on hand two standard solutions, one of which contains 0.01 mg. of uric acid per cc., while the second contains 0.02 mg. of uric acid in 5 cc. of solution. The second standard is the one commonly employed, but the first may occasionally be of service, and is valuable in instances where it is desired to use the Folin-Wu procedure for comparison of results by the old and new procedures. For the preparation of the first standard, 25 cc. of the phosphate standard solution (containing 5 mg. of uric acid) are measured into a 500 cc. volumetric flask, and the flask is about half filled with distilled water. 25 cc. of dilute hydrochloric acid (1 volume of concentrated acid diluted to 10 volumes with water) are added, and the solution is diluted

to 500 cc. This solution contains 0.01 mg. of uric acid in 1 cc. For preparation of the second standard (the one which is most frequently employed) the procedure is the same, except that instead of starting with 25 cc. of the phosphate solution, 10 cc. are employed and diluted after acidification exactly as for the other standard. It should be remembered that these standard solutions should be freshly prepared once in 2 weeks.

In connection with the use of the standard solution it should be pointed out that in the new method, as with most colorimetric methods, results are most accurate when standard and unknown correspond closely in depth of color. Using 0.02 mg. of uric acid as standard, results are satisfactorily exact when the unknown reads between 10 and 24 mm., when the standard is set at 15 mm. This represents a quite satisfactory range for bloods, as one standard is applicable for bloods containing from about 2.5 to 6 mg. of uric acid per 100 cc. With 2 mg. of uric acid results may be about 10 per cent too high; *i.e.*, we might obtain 2.1 or 2.2 mg. of uric acid per 100 cc. instead of the true value of 2 mg. Absolute results, where desired, can, of course, be obtained for bloods on the outer limit of any standard by a repetition of the determination, using a closer standard. Increased accuracy cannot be obtained by changing the dilution of either the standard or unknown after the reaction is completed. The determination is so simple, and the quantity of material required so small, that it is no hardship to repeat determinations on the few bloods which fall outside the limits of 2 to 6 mg. per 100 cc. In repeating the determination on such bloods it is desirable to correct by using more or less of the blood filtrate, as indicated by the determination, with corresponding change in addition of water to make a total volume of 10 cc. before addition of cyanide and reagent as described below. It is safer in such cases to repeat with 5 cc. of filtrate, using 0.01 mg. of uric acid in the standard. Where more than 5 cc. of blood filtrate are employed the reading should be made promptly, in order to avoid the development of turbidity.

It should be stated that the method described below yields a slight trace of color in a blank determination. To those who have been in the habit of reading the Folin-Wu uric acid solution this color may appear a serious matter. In the present method, where the color developed is relatively so much more intense, the

color from a blank is negligible, inside the limits above mentioned, since it is present in both standard and unknown.

The blood is precipitated with tungstic acid as described by Folin and Wu (3), the blood being allowed to stand at least 10 to 20 minutes after adding the tungstate and sulfuric acid, before filtration. This tends to insure complete protein precipitation. The use of excess of acid in the precipitation is to be avoided.

5 cc. of the water-clear filtrate (representing 0.5 cc. of blood) are transferred to a test-tube[2] and 5 cc. of water are added. The standard solution, containing 0.02 mg. of uric acid (prepared as described above), is placed in another tube and the volume likewise made up to 10 cc. To both standard and unknown are added 4 cc. of 5 per cent sodium cyanide solution[3] containing 2 cc. of concentrated ammonia per liter.[4] To each tube is then added 1 cc. of the arsenic phosphoric acid tungstic acid reagent. The contents of each tube should be mixed by one inversion immediately after addition of the reagent, and placed immediately in boiling water, where the tubes should be left for 3 minutes after immersion of the last tube, but the time elapsing between immersion of the first and last tubes should not exceed 1 minute. There will be no difficulty here in getting the tubes all into the hot water within 1 minute, unless it is attempted to run more than about five unknown solutions in one series. A 3 minute sand-glass is very convenient in connection with the heating. After the 3 to 4 minute heating the tubes are removed and placed in a large beaker of cold water for 3 minutes and read in a colorimeter against the standard as soon as may be convenient. Long standing before reading may lead to development of turbidity. It is best to read the solution within

[2] As will be seen from the description of the method the test-tubes used need not be graduated, since no dilution is ordinarily made prior to reading in the colorimeter. The test-tubes employed should be of uniform diameter (18 to 20 mm.).

[3] On account of the high toxicity of cyanide solutions they should never be handled in pipettes, but should always be measured from a burette. The reagent is also best measured from a burette.

[4] Some cyanide solutions were found to yield more color in blank determinations than did others. Experiments on this point showed that it is desirable to have a trace of ammonia present in the cyanide solution. It is desirable to prepare the cyanide solution fresh once in 2 months.

5 minutes after removing from the cold water. Where this is done we have never encountered turbidity. Where a large number of bloods are to be analyzed it is best to run not more than four at a time with one standard. This provides for greater uniformity in handling, does not cool the bath down too much, and makes it easy to finish reading before any turbidity may develop.[5]

A few words of explanation concerning the adoption of heating in connection with the uric acid determination may be desirable. We adopted the immersion in hot water for a few minutes after a very careful study had demonstrated that it is not possible to obtain conditions where the reaction goes to even approximate completion in less than 15 minutes at room temperature. Such standing may lead to development of turbidity, and we have found it much better to adopt the short period of heating, which is certainly no hardship. Prolonged heating is to be avoided, since the color will fade under such conditions. There is no difficulty whatever if the tubes are placed in water which is within 10° of boiling and left there for 3 to 4 minutes, and then immersed in cold water as described above.

Calculation.—Employing the standard solution containing 0.02 mg. of uric acid and using 5 cc. of the 1:10 blood filtrate, the calculation for the uric acid content of the original blood is as follows:

$$\frac{S}{R} \times 4 = \text{mg. of uric acid per 100 cc. of original blood}$$

in which S represents the height of the standard solution in millimeters, and R the reading of the unknown solution. If instead of using 5 cc. of blood filtrate in the determination, 2.5 or 10 cc. are employed, the final figure is multiplied or divided by 2 accordingly.

[5] Excess of potassium salts in blood filtrates is undesirable in both the uric acid and the creatinine determinations. It would probably be advantageous for these determinations to employ sodium oxalate instead of the potassium salt to prevent coagulation. There is no apparent advantage in the almost universal habit of using potassium oxalate as an anticoagulant for blood.

Discussion of the Results by the New Method.

In Table I are given the results of determination of uric acid by the new method upon fifty samples of human blood.[6] We have also included in the table figures for the uric acid on the same blood samples obtained by a slightly modified Folin-Wu procedure, together with figures for the non-protein nitrogen. Sufficient blood was not available for comparison by the older silver magnesium precipitation method, but Folin and Wu have stated that their method essentially duplicates the results by the older procedure. In using the Folin-Wu procedure our technique has been identical with that described by these writers up through the decomposition of the precipitate by means of 10 per cent sodium chloride in 0.1 N hydrochloric acid. From this point our procedure differed from that described by Folin and Wu, because these writers advocated the use of a sulfite-containing standard, which has not appealed to us. The use of the sulfite standard, together with the dilution employed by Folin and Wu led to such weak final colors that we were unable to read them. We shall describe our exact technique from the point of precipitation of the uric acid, as it may prove useful to others in connection with checking up results by the new method upon any particular samples of blood.

In using the Folin-Wu technique we have invariably employed 20 cc. of filtrate for the precipitation, except where the blood uric acid exceeded 6 mg. per 100 cc. In such cases 10 cc. of the filtrate were used. The measured volume of filtrate, contained in a centrifuge tube, is precipitated by means of the silver lactate-lactic acid solution[7] as described by Folin and Wu. After cen-

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[6] These bloods were obtained from the pathological laboratories of the Roosevelt Hospital through the courtesy of Dr. Wm. G. Lyle, Director. We are also indebted in this connection to Mr. Harry Osterberg, whose figures for non-protein nitrogen on these bloods we have included in the table.

[7] Silver lactate purchased in the market is apt to contain considerable amounts of reduced silver, and the product is quite expensive, and often difficult to obtain. We have prepared our silver lactate-lactic acid solution starting from silver nitrate, as follows: Dissolve 22.5 gm. of silver nitrate in about 500 cc. of water in a liter stoppered cylinder. Add an excess (about 60 cc.) of approximately 5 N sodium or potassium hydroxide

trifugation the residue in the tube is *very thoroughly* stirred with the acid sodium chloride solution, using 1 cc. where 10 cc. of blood filtrate were precipitated, and 2 cc. where 20 cc. were used. A volume of water is now added to the centrifuge tube[8] which will bring the total volume of the solution to 10 cc. where the quantity of uric acid expected does not exceed 0.08 mg., or to 12 cc. where a quantity between 0.08 and 0.12 mg. is expected. (These figures were selected because they represent the maximal concentration obtainable where we employed the standard solution containing 0.01 mg. of uric acid per cc.) The contents of the centrifuge tube are again thoroughly stirred, and after centrifugation are poured as completely as possible into a clean dry test-tube. In two other tubes quantities of the standard solution previously described (containing 0.01 mg. of uric acid per cc.) are measured to give a total uric acid content in one tube of 0.05 mg. and in the second of 0.08 mg. of uric acid. If high uric acid is expected it is desirable to have a third standard containing a total of 0.1 mg. of uric acid. 2 cc. of the acid sodium chloride solution are added to each, of the tubes, and the volume made to 10 cc. for the weaker standards, and left at 12 cc. for the stronger standard. To each standard and unknown tube are now added 3 drops of 5 per cent sodium cyanide solution, and 1 cc. of the Folin-Denis uric acid reagent, followed by 2 cc. of 20 per cent sodium carbonate solution. The contents of the tubes are mixed by a single inversion, and read promptly in the colorimeter, setting the standard at a height of 20 mm. Should any of the unknown

solution, then add water to about 1 liter and shake. Allow to stand for a few minutes and decant the supernatant fluid. Add distilled water to about 1 liter, shake, and pour off the supernatant fluid after a moment or two. Repeat this washing by decantation until the wash water fails to react alkaline to litmus paper. The total washing process need not take longer than 10 minutes. After the last decantation make up to a volume of about 200 cc. with distilled water, and add 35 cc. of lactic acid (sp. gr. 1.2). Shake thoroughly and dilute to 500 cc. Filter through a dry folded filter, returning the first portions until the filtrate appears as clear and colorless as distilled water. Filter the entire solution through this filter, and preserve in an amber, glass-stoppered bottle.

[8] In the description it is assumed that either the 10 or 20 cc. of filtrate has been precipitated in one centrifuge tube. Where two tubes have been employed one-half the indicated quantity of chloride solution and of water should be added to each of the two tubes.

tubes having a dilution of 10 cc. match the 0.1 mg. standard more closely than any of the weaker standards, this comparison may be made after adding 2 cc. of water to the unknown tube. In employing the Folin-Wu procedure it should be remembered that unknown and standard solutions must match quite closely for satisfactory results.

We shall now discuss the comparative figures obtained by the two methods. For facilitating this comparison we have tabulated in Column 4 the difference by the two methods in milligrams of uric acid in 100 cc. of blood, using the Folin-Wu figures as standard. Thus where the new method gives results higher than the old, figures in Column 4 are preceded by a plus, and where lower, by a minus sign.

Even a casual inspection of Table I reveals that the new method tends to give higher results than does the Folin-Wu procedure, at least on those bloods which contain less than 50 mg. of non-protein nitrogen per 100 cc. Thus out of the total of fifty bloods, we find that in thirty-one, or 62 per cent, the new method yields higher figures than the other. This fact in itself could not, however, lead us to question the accuracy of the new method, since in the new procedure we have eliminated precipitation, decomposition, and transfer of the final solution, which would theoretically tend to cause slight losses. For a proper consideration of the differences by the two methods it is, therefore, more correct to take account only of differences which exceed, let us say, 0.5 mg. per 100 cc. of blood, since duplicates by the same method will frequently vary by this amount, and plus differences up to the 0.5 figure can properly be credited in favor of the new method. Out of the total of fifty bloods we find that there are twenty, or 40 per cent, in which the new method exceeds the old by more than 0.5 mg. per 100 cc., eight (16 per cent) in which the figures lie between 1 and 1.5 mg., and none in which the new method exceeds the old by as much as 2 mg. of uric acid per 100 cc. of blood. We shall return later to a discussion of the higher figures by the new method.

From a general standpoint it would appear that if there are no cases where the figures by the new method exceed those by the old by as much as 2 mg. per 100 cc., that very probably the question is a theoretical rather than a practical one. But a very

TABLE I.

Results Obtained by the New Method Compared with Those Obtained by a Slightly Modified Folin-Wu Procedure, on the Same Samples of Human Blood.

Sample No.	Per 100 cc. of blood.			
	New method.	Folin-Wu method.	Difference, Folin-Wu figures as standard.	Non-protein nitrogen.
	mg.	*mg.*	*mg.*	*mg.*
1	4.4	5.2	−0.8	103
2	2.5	2.6	−0.1	33
3	3.6	2.5	+1.1	38
4	3.2	3.0	+0.2	41
5	3.2	3.2		60
6	3.4	5.0	−1.6	33
7	6.6	6.6		69
8	4.0	3.2	+0.8	30
9	5.2	4.8	+0.4	33
10	3.5	3.2	+0.3	30
11	13.6	15.7	−2.1	197
12	2.4	2.0	+0.4	28
13	4.0	2.4	+1.6	33
14	2.8	2.3	+0.5	29
15	2.6	1.8	+0.8	36
16	4.2	3.1	+1.1	204
17	2.6	2.4	+0.2	40
18	4.0	4.4	−0.4	45
19	2.4	2.3	+0.1	46
20	4.1	3.4	+0.7	39
21	0.8	1.2	−0.4	27
22	2.7	2.1	+0.6	41
23	2.8	2.0	+0.8	35
24	3.0	2.0	+1.0	36
25	6.0	6.6	−0.6	105
26	5.6	6.0	−0.4	201
27	3.7	3.4	+0.3	45
28	4.3	3.0	+1.3	41
29	4.2	4.0	+0.2	37
30	3.6	4.2	−0.6	43
31	2.9	2.7	+0.2	37
32	3.6	2.7	+0.9	38
33	4.6	3.8	+0.8	42
34	2.8	2.9	−0.1	36
35	3.8	2.9	+0.9	28
36	3.6	2.5	+1.1	44

TABLE I—*Concluded.*

Sample No.	Per 100 cc. of blood.			
	New method.	Folin-Wu method.	Difference, Folin-Wu figures as standard.	Non-protein nitrogen.
	mg.	*mg.*	*mg.*	*mg.*
37	2.7	2.4	+0.3	28
38	9.5	10.0	−0.5	260
39	3.0	3.4	−0.4	29
40	4.6	3.5	+1.1	43
41	4.5	4.0	+0.5	57
42	3.0	3.0		30
43	4.0	5.0	−1.0	50
44	4.2	3.5	+0.7	36
45	3.2	1.5	+1.7	27
46	10.9	11.7	−0.8	277
47	4.7	4.5	+0.2	29
48	3.2	3.4	−0.2	36
49	2.9	2.3	+0.6	30
50	5.2	3.8	+1.4	45

crucial question arises at this point. If the tendency to higher results by the new method is due to an interfering substance, and this substance tends to increase in the blood in conditions of kidney inefficiency, then we would have to conclude that the new method could not be of service for determining uric acid. This point seems adequately covered by the results on the bloods which showed 60 mg., or more, of non-protein nitrogen per 100 cc. (Table I; Nos. 1, 5, 7, 11, 16, 25, 26, 38, 41, and 46). These bloods cover a wide range of nitrogen retention as indicated by figures from 60 to 277 mg. of non-protein nitrogen per 100 cc. There is excellent agreement by the two methods in five out of the ten bloods. In three of the remaining five, the new method gives *lower* results than the old, ranging from 0.4 to 2.1 mg. per 100 cc. of blood. In only one case of a blood with marked accumulation of non-protein nitrogen does the new method yield an appreciably higher figure than the old; *viz.*, 1.1 mg. per 100 cc. in Blood 16.

We believe that there is definite basis for assuming that the lower figures which the new method gives in the majority of the retention cases are the more correct. Theoretically the lower

figures should be regarded as correct, and it can be shown that there is experimental evidence in favor of this view.[9]

The silver lactate precipitation of uric acid from dilute blood filtrates as suggested by Folin and Wu is a remarkable example of accomplishing the seemingly impossible, and is not at all a simple ordinary precipitation as might be inferred from reading a description of the method.   Folin and Wu provide for precipitating uric acid from the blood filtrate where the actual concentration of the uric acid may be even less than 1 mg. in a liter, or 1 part in 1,000,000.   Even the most insoluble salts known, such as silver iodide cannot be simply precipitated from such great dilution. Furthermore, if one adds the silver lactate solution to pure uric acid solution of five or even ten times the concentration existing in normal blood filtrates there is no visible precipitation.   Yet the fact remains that the uric acid is actually removed from the blood filtrate by the silver lactate precipitation.   Hence we must infer that the uric acid (probably as the silver salt) is adsorbed by, or in some way dragged down with, other substances simultaneously precipitated.   Probably chloride, oxalate, and tungstate (all of which are usually present in the Folin-Wu blood filtrate) contribute to this removal of uric acid from these dilute solutions. It is, however, doubtful upon theoretical grounds whether a precipitation accomplished in this way can be highly specific for any one compound.   It is easy to demonstrate that in the case of blood filtrates other substances are carried down with the uric acid, and what is important for our present discussion, that most of the color-yielding substances (in the uric acid reaction) are so precipitated.   Here we encounter findings which will impress many readers as not only difficult of interpretation, but as probably involving false assumptions or incorrect work.   Yet the present writer has worked on this question in considerable detail and can offer only the conclusions given below, together with the facts upon which they are based.

It is notable that Folin and Wu, instead of dissolving their precipitated uric acid in cyanide, and making the determination directly upon this solution as they do in the case of the urine,

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[9] Uric acid added directly to blood filtrates is recovered very satisfactorily in the new method, indicating that there is nothing in blood filtrates which inhibits the development of the color due to uric acid.

take the extra steps of decomposing the precipitate with a chloride solution and centrifuging off the silver chloride. No explanation is offered by Folin and Wu as to why this apparently unnecessary procedure is adopted in the case of the blood. But if we attempt to get along without it, we immediately get into trouble. If the "silver urate" precipitate is dissolved in enough cyanide to readily bring it into solution (about 10 drops of 5 per cent solution) and an equal quantity of cyanide added to the standard solution (employing, of course, the old phosphotungstic acid reagent for the final color production) we commonly obtain results which are from 150 to 300 per cent too high.

The high results under such conditions might be due to one of three factors: (1) The silver present in the solution where the precipitate has been directly dissolved might be responsible for the high result; or (2) other color-yielding substances may have been precipitated from the blood which dissolve unaltered in the cyanide, but which are decomposed by treatment with acid sodium chloride so that they no longer yield color with the uric acid reagent; or, (3) other color-yielding substances may have been precipitated from the blood which pass into solution when the whole precipitate is directly dissolved, but which remain in the precipitate when treatment with acid sodium chloride is employed.

It can be proved beyond a reasonable doubt that neither of the first two suppositions can explain the high results. If the same silver lactate solution employed in the precipitation is added to a standard solution of uric acid it has practically no effect upon the reading. If a few drops (1.5 drops for each cc. of solution) of 10 per cent sodium tungstate solution (the one employed in the original precipitation of the blood) are added to a standard solution of pure uric acid of about the concentration found in blood filtrates, and this solution is then treated with the silver lactate, the precipitate centrifuged off, and the procedure concluded as for the blood, it will be found that the uric acid taken is recovered almost exactly whether the precipitate be decomposed by the acid chloride solution, or whether it be dissolved directly in cyanide.

These results seem to demonstrate quite conclusively that the high results where cyanide is employed directly on blood filtrates

are not due to the presence of the silver.   The demonstration is of importance and shows that the high figures where cyanide is employed directly, are not due to finely divided metallic silver (which would, of course, reduce the phosphotungstic acid reagents).   That metallic silver plays no part in this connection is also indicated by the light color of all the precipitates obtained with the blood.   The merest traces of metallic silver in a mixture will darken it perceptibly, and this effect is absent from the precipitates with properly prepared and kept silver lactate solution.

The second possibility above suggested, *viz.* that the high results may be due to a compound which is destroyed by the acid solution, is shown not to be correct by the fact that if the precipitate from blood is stirred with acid sodium chloride and then the entire precipitate dissolved in cyanide the results are still two or three times too high.

By exclusion then, we arrive at the conclusion that the silver lactate precipitates other compounds from blood filtrates which give the uric acid reaction with the old reagent.   This conclusion is brought practically to the point of certainty by noting that the figures obtained by dissolving the "silver urate" directly in cyanide increase with increase of total interfering substances in the blood. Indeed for many bloods it seems that practically all the color-yielding substance present in the blood, uric acid, and non-uric acid, is precipitated by the silver lactate solution.   Illustrative results in this connection for a few bloods are given in Table II. In order to show the much greater specificity of the new process for uric acid determination, figures obtained by applying the new process directly to the same blood filtrates, are also included.

It seems clear from the results reported in Table II that there is a direct relationship between the total color-yielding compounds in the blood, and the color obtained where the "silver urate" is dissolved directly in cyanide.   We must, therefore, conclude that while these substances may be largely precipitated by silver lactate in lactic acid, Folin and Wu found a means of decomposition for the precipitate which effects a remarkable separation between the uric acid and the other color-yielding compounds. It seems quite inexplicable to the present writer how, upon theoretical grounds, this separation can be effected, but the facts seem to admit of no other explanation.   It seems reasonable,

however, to assume that the tendency to higher results by the Folin-Wu method for the bloods high in non-protein nitrogen is best explained upon the assumption that some of the precipitated non-uric acid color-yielding material may be set free during the decomposition of precipitates containing excessive quantities of this material. Certainly it appears that the lower results for such bloods by the new method are probably more nearly the correct ones.

The tendency to somewhat higher figures by the new method for ordinary bloods as exemplified in Table I can scarcely be definitely explained at present. It is possible that the excess

TABLE II.

Showing the results obtained with human bloods when the "silver urate" precipitate is dissolved directly in cyanide solution. Comparative figures are given by the modified Folin-Wu decomposition method, and by the old and the new reagents applied directly to the blood filtrate.

Sample No.	Per 100 cc. of blood.				
	Folin-Wu regular decomposition of precipitate.	"Silver urate" dissolved directly in cyanide.	Old reagent applied directly to blood filtrate.	New process applied directly to blood filtrate.	Non-protein nitrogen.
	mg	mg.	mg.	mg.	mg.
1	1.5	5.2	10.1	2.3	43
2	10.2	27.4	28.1	9.5	281
3	2.4	6.5	7.6	3.4	38
4	2.7	5.6	7.8	2.9	37
5	2.5	5.0	7.4	3.1	39
6	15.7	19.1	20.5	13.6	197

figures represent an effect of some interfering substance which shows no tendency to increase with kidney inefficiency. This explanation is unlikely, especially since the difference between the two methods tends to disappear or is reversed as soon as the uric acid by the Folin-Wu method approaches a figure of 3.5 to 4.0 mg. The only evidence that we have that the figures by the new method represent essentially only uric acid in the bloods showing the discrepancy as well as in the others, is the fact that where the determinations are made by letting the filtrates stand in the cold, so that the maximal color develops slowly, it is found that where standard and unknown contain approximately the same

amounts of the reacting substance as measured by the final color, results are practically identical whether the readings be made early or late in the process of color development. This similarity in rate of color development in standard and unknown lends support to the view that the figures represent uric acid only. Conversely, since the exact conditions of salt content, acidity, etc., and other factors which may influence the adsorption precipitation of the silver urate in the blood filtrates cannot be readily duplicated upon solutions totally free from uric acid for tests upon complete recovery, there is some question as to whether the small increment of uric acid shown in the new direct method may not be lost in some way in the precipitation and decomposition method.

Another possible explanation which suggests itself concerning these differences is that in these bloods we may be dealing with the presence of some second form of uric acid—combined or altered in some way so that it reacts more strongly in the new method than in the old. We investigated this possibility, and soon became convinced that it deserves no consideration. When we applied the new process to the solution obtained by decomposing the silver precipitate as in the Folin-Wu method we invariably obtained figures identical with or *lower* than where the old reagent and procedure were employed. In no instance where the new and the old processes have been applied actually to portions of the *same solution*, whether the blood filtrate directly, the solution after sodium chloride decomposition, or the solution obtained by dissolving the silver precipitate directly in cyanide, have we obtained higher figures by the new process. They have usually been appreciably lower.

Hence it seems that there can be no question of a second form of uric acid which reacts differently with the two procedures in these bloods.

It seems that an impartial study of Table I should lead to the adoption of the new procedure for general work.[10]   The method

[10] If one prefers to continue using the Folin-Wu precipitation and decomposition method, the new process can, of course, be applied to the solution thus obtained. 5 or 10 cc. of filtrate are precipitated and decomposed as in the Folin-Wu method, the volume being made to 10 cc. with water before the final stirring and centrifugation. The clear solution obtained after centrifuging is poured as completely as possible into a test-tube, and the process concluded as in the regular new method.

is more expeditious and requires the use of less blood than does any other procedure. It is simple and gives a satisfactory depth of color and is far safer in the hands of many analysts. The differences by the two methods are in many instances negligible, and in only one or two instances out of the fifty analyses reported, would acceptance of the new figures suggest a different interpretation as regards uric acid abnormality or retention. In one instance, Blood 13, the new method gives a figure above the accepted normal, while in another, No. 43, the interpretation would be reversed if the new figures are accepted. In the latter case the figure by the new process is almost certainly more correct. The difference by the two methods chiefly affects the bloods where the Folin-Wu method gives 2.5 mg. or below. Thus we find that out of the fifty bloods, the Folin-Wu method gives a figure of 2.5 mg. or below, per 100 cc. for fifteen bloods, while only five bloods give similarly low figures by the new process. Where the new process is used it is probable that the commonly accepted figures should be increased by about 0.5 mg. per 100 cc., so that the usual normal figures would probably be about 3.0 mg., while no evidence of definite uric acid retention would be evident before a figure of 4.0 mg. or over per 100 cc. was reached. The question as to whether the figure 4.0 mg. per 100 cc. of blood represents a true pathological retention must remain open, just as it is for quite similar figures by the old methods. Such border-line questions can be answered only by extensive data.

A few words covering the quantity of blood filtrate necessary or desirable to carry out the new procedure may be of value. As the method is above recommended it will be noted that we finally heat a rather large volume (15 cc.) of a very dilute solution. This solution may contain as low a concentration of uric acid as 1 part in 1,500,000 parts of solution or even less, and accurate results can still be obtained. It may seem that it would be better to carry out the reaction with more concentrated solutions, or with smaller volumes. We have found that higher concentrations are not so desirable, as turbidity may occur, and the results as a whole are not so uniform. It is, of course, perfectly possible to cut everything in half in the process as above described, using 2.5 cc. of filtrate, and having an ultimate volume of 7.5 cc. during the heating. This is permissible where circumstances may

require it, but the writer believes that the larger volumes are commonly measured with greater accuracy, and since 0.5 cc. of blood is certainly not excessive for a uric acid determination, it seems better to recommend starting with that equivalent unless there are reasons to the contrary.

There are, of course, certain instances where it may be desirable or necessary to work with very minute quantities of blood, such as can be obtained readily by puncture. In the case of infants, or where repeated examinations on the same individual are to be made, the use of blood obtained by puncture may be essential. For this reason a modification of the new procedure is here described which might perhaps be properly called an "ultra-micro" method, since it can be used to determine as little as 0.002 mg. of uric acid, and yields results very comparable with those obtained by the procedure described earlier in this paper. The modification requires the use of only 0.2 cc. of blood (0.1 cc. of filtrate), and is carried out as follows:

0.2 cc. of blood is carefully pipetted into a narrow pointed centrifuge tube. 1.4 cc. of water are added and the mixture is stirred with a fine glass rod. 0.2 cc. of 10 per cent sodium tungstate solution is added, followed by 0.2 cc. of 0.75 N sulfuric acid, and the mixture is thoroughly stirred and allowed to stand for 10 minutes. The tube is then centrifuged at high speed for a few moments and 1 cc. of the clear supernatant fluid is pipetted into a long narrow (1 cm.) test-tube. 1.8 cc. of 2.8 per cent sodium cyanide solution (containing 1.5 cc. of concentrated ammonia per liter) are then added, followed by 4 drops (0.2 cc.) of the arsenic phosphotungstic acid reagent. The mixture is then treated as is the unknown in the regular process, and can be compared with the 0.02 mg. regular standard solution. The 3 cc. of colored solution are readily read in the narrow form cup of the Bock-Benedict colorimeter up to a height of about 24 mm.

In using this modification it is, of course, essential that calibrated pipettes be employed, and that all the measurements be made with great care. Under such conditions we have obtained surprisingly close duplicates between the two modifications. Of ten bloods analyzed by both methods the maximal difference was 0.6 mg. of uric acid per 100 cc., and in six of the samples the variation did not exceed half of this figure.

. In conclusion one or two points of general interest in connection with the new process may be mentioned. The increased specificity of the new procedure as regards uric acid lies probably chiefly in the reagent employed and only partly in the use of cyanide instead of carbonate for development of the alkalinity. A corresponding, though not so great, increase in color is obtained with the old phosphotungstic acid reagent through the use of large quantities of cyanide, but the reaction thus obtained is not very specific for uric acid. The cyanide acts in a dual capacity in causing the increased color. It has an effect upon the reaction, probably through combination with the uric acid as exemplified in the older procedures, and it is superior to carbonate as an alkali, because it causes a much slower decomposition of the reagent than does the stronger alkali, and hence gives more time for the reaction at the critical point when the linkage of the complex tungstic compound is being broken. That this view is correct is rendered probable by a fact observed by the writer some years ago that the use of borax in hot solution would markedly increase the color yield in the uric acid determination. The results were not reported because the color developed was not closely proportional to the quantity of uric acid present. In this instance the specificity of the reaction was not appreciably increased, although the color yield for a given quantity of uric acid was greater than in the carbonate process.

It is to be noted that the new process described in the present paper is now recommended only for application to the Folin-Wu filtrates from human blood. Studies of the new process under certain other conditions of precipitation, and for other bloods, are being carried on.

We shall shortly report upon the application of the new procedure to the determination of uric acid in urine.

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A COLORIMETRIC METHOD FOR THE DETERMINATION OF SUGARS IN NORMAL HUMAN URINE.

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The colorimetric principle underlying the method of Folin and Wu[1] for the determination of sugar in blood should prove equally useful for the determination of sugar in normal urines provided that a suitable process could be found for removing substances which can interfere. The practicability of the process would necessarily depend on the preliminary treatment required for the removal of creatinine, uric acid, and other materials which might have considerable reducing power.

The process finally adopted is extremely simple, and from the very extensive use which we have made of it during the past year we have become convinced that it meets all practical requirements. The preliminary treatment consists only of shaking the urine with "Lloyd's alkaloidal reagent,"[2] a concentrated fullers' earth. This reagent removes most of the coloring matters together with the uric acid, creatine, and the creatinine, yet, unlike all or most effective charcoals, does not take away the sugar.

It is not necessary that every trace of creatinine should be removed for relatively considerable amounts have absolutely no effect on the sugar method of Folin and Wu. That this is the case was shown in connection with the application of the method to blood.

The process is as follows: To 5 cc. of urine add 5 cc. tenth normal sulfuric acid and 10 cc. of water. Add 1.5 gm. of Lloyd's reagent and shake gently for 2 minutes. Filter. 2 cc. of the filtrate are the usual amount used for the sugar determination. The above mentioned dilutions are for concentrated urines. With more dilute ones, one takes 10 or 15 cc. and reduces the amount of water taken.

The shaking with Lloyd's reagent should not be continued longer than 2 minutes because the reagent is gradually dissolved by the acid and because longer shaking does not take out any more. The dissolved aluminate from the reagent does not disturb the determination at any stage.

The colorimetric determination of the sugar in the filtrate is made in exactly the same manner as in the case of blood filtrates.

For the determination of the total sugar we hydrolyze as follows: To 10 cc. of the filtrate obtained after shaking with Lloyd's reagent add 1 cc. of 10 per cent hydrochloric acid and heat in boiling water for 75 minutes. This heating should be done in test-tubes graduated at 20 cc.—for purposes of subsequent dilution. After hydrolysis, cool thoroughly and neutralize with normal sodium hydroxide. Phenolphthalein may be used as indicator, if desired, but is not necessary as the cloud produced from the material dissolved out of Lloyd's reagent furnishes an adequate indicator of the degree of neutrality required. Add the alkali until the cloud so formed does not disappear on shaking.

Dilute the neutralized hydrolysate to the 20 cc. mark. Then add a small pinch of Lloyd's reagent and invert half a dozen times. This is for the purpose of removing most of the coloring matter formed during the hydrolysis. 2 cc. of this more dilute filtrate are usually a suitable amount to take for this determination also.

The standard sugar solutions to be used are the same as for the blood; namely, such as contain 1 and 2 mg. of glucose per 10 cc. A few remarks concerning such standard sugar solutions may appropriately be introduced here. It is a common experience that whereas moderately concentrated sugar solutions can easily be preserved the dilute ones often deteriorate. We suspect that the cause for this deterioration is alkali given off from the containers rather than destruction by microorganisms.

At all events, we have found that the dilute as well as the concentrated solutions keep perfectly in 0.3 per cent of benzoic acid and we, therefore, make the original stock solution containing 1 per cent of glucose by means of 0.3 per cent benzoic acid solution. The same benzoic acid solution is then used, instead of water, for the preparation of the dilute standard solutions.

SOME NEW OBSERVATIONS AND INTERPRETATIONS WITH REFERENCE TO TRANSPORTATION, RETENTION, AND EXCRETION OF CARBOHYDRATES.

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CONTENTS.

Review of the More Important Literature with Some Comments.

Many investigations have been reported representing the simultaneous determination of certain waste products in blood and in urine, the general purpose of which has been to elucidate how the concentration of these products in the blood determines the rate at which they are eliminated through the kidneys. A fairly large number of analogous investigations have been made with "foreign" substances and also with certain food products, particularly glucose. Glucose has heretofore been almost the only food material so investigated, and for many obvious practical reasons. The field as a whole is an interesting one, for it has to do not only with the activity of the kidneys but with other equally important problems, notably the power of the tissues to abstract from the blood with a high degree of speed and completeness many products such

as uric acid, creatine, amino-acids, and probably many other substances, besides the water-soluble food materials.

In the present paper we deal mainly with the rise and fall of sugar in blood and urine following the intake of glucose and other carbohydrates.

There is a very large literature both old and new on this subject, yet both as to the essential facts and still more as to the interpretations the modern literature is almost as conflicting as the old. Compare for example the opinion of Shaffer[1] (1921) on the sugar of normal urine with that of Benedict[2] published in 1918. The one virtually denies the presence of glucose in normal urines, the other almost implies that all are more or less on the verge of becoming really diabetic. When it comes to such concepts as limits of sugar assimilation, sugar tolerance, and the renal threshold for sugar, a careful sifting of literature is greatly needed.

Our original purpose was to include such a sifting of the literature in this paper, but it soon became apparent that to do so would take up too much space, and we have confined ourselves to brief summaries of only the more important investigations.

The existence of a relationship between the concentration of sugar in the blood and the appearance of sugar in the urine was clearly recognized by Claude Bernard,[3] and was brought out in connection with the experimental glycosuria produced in dogs by his sugar puncture as well as by the administration of curare. Claude Bernard even gives definite figures (224 to 260 mg. per 100 cc. of arterial blood) for the blood sugar concentration below which no glycosuria occurs; and in another connection he observes that the blood sugar may sometimes rise to 300 mg. without causing the "healthy" kidneys to eliminate any sugar. The latter figure is still cited in some text-books.[4] Claude Bernard had thus clearly in mind the renal threshold concept for sugar, although he did not use the term. Claude Bernard did not specifically deny the presence of minute traces of sugar in all normal urines; he simply omitted to express a conviction on that problem.

[1] Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, 1920–21, xlv, 365.

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[3] Bernard, C., Leçons sur la diabète et la glycogénèse animale, Paris, 1877.

[4] Cushny, A. R., The secretion of the urine, London, 1917.

Pavy,[5] though originally a pupil of Claude Bernard, came to entirely different conclusions concerning the conditions and rela-· tionships existing between the sugar of blood and the sugar in the urine. Pavy held that the kidneys are completely permeable for, glucose, that normal urine always contains traces of sugar, and that the reason why it does not contain more is the fact that the blood contains only similar insignificant traces of free sugar. The major part of the so called blood sugar Pavy regarded as a post-, mortem phenomenon, for in life the absorbed sugar is held in combination with protein material in non-dialyzable form.

It is easy enough to comprehend why Pavy never could give·up· the concept of some chemical glucose-protein combination in blood as the mechanism by which the escape of all but insignifi-· cant traces of sugar into the urine are prevented. While he never succeeded in proving the correctness of his theory he nevertheless did prove by his discovery of the glycoproteins that sugar-protein combinations do exist to a limited extent. Definite proof that the Pavy-Lépine interpretation is incorrect, that the blood sugar exists in free, diffusible form, was finally furnished through the compensation dialysis experiments of Michaelis and Rona.[6] The retention of a normal amount of blood sugar as well as the failure of such retention at a given concentration, above the sugar threshold, must therefore presumably be a feature of the kidney activity alone, and this is indeed the conclusion arrived at by Hamburger and Brinkman in their highly interesting work on the excretion of sugar urines by the glomeruli of frog's kidneys.

Hamburger's investigations are interesting not only in connection with the problem of why the kidneys eliminate glucose when the glucose of the plasma exceeds a certain concentration (the threshold), but the results are also extremely interesting as illustrations of the selective activity of kidneys, or of phenomena simulating such activity.

By varying the calcium and the sodium bicarbonate content of the modified Ringer's solutions with which the frog's kidneys were perfused the activity of the kidneys to added glucose could be varied from complete permeability to complete impermeability for glucose in concentrations corresponding to those found in frog plasma. In response to an excess of sugar, 0.2 to 0.3 per cent, the impermeability of the kidneys broke down. ·

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[5] Pavy, F. W., On carbohydrate metabolism, London, 1906; *J. Physiol.*, 1899, xxiv, 479; 1900–01, xxvi, 282; *Lancet*, 1908, 1499, 1577, 1727.

[6] Michaelis, L., and Rona, P., *Biochem. Z.*, 1908, xiv, 476.

The conditions resulting in the complete retention of added glucose had no such effect on other sugars. When mixtures of glucose and levulose or glucose and lactose were used in suitable concentrations, the levulose or the lactose was passed quantitatively into the "urine," while glucose remained in the perfusing fluid. Similar results representing varying degrees of separation were obtained with a number of other sugars.

One must necessarily hesitate about the extent to which one is willing to accept the theoretical discussion given by Hamburger, but the data are at least interesting and seemingly represent several years of patient research.[7]

A characteristic feature of the more recent investigations has been the establishment of the necessary data with a greater degree of precision than was possible by the methods of Pavy or any of the older investigators. Jacobsen[8] in Copenhagen was probably the first who on the basis of a modern method for the determination of blood sugar (Bang's) attempted to find the blood sugar level at which positive (clinical) tests for sugar are obtained in the urine. He gave 100 gm. of glucose to each of fourteen normal persons (either before, or 2 to 3 hours after breakfast) and determined the blood sugar every 15 minutes and tested the corresponding urines for sugar, mostly by Almén's test. In eight of the fourteen subjects he obtained positive alimentary glycosuria, and he found that the glycosuria began when the blood sugar rose above 160 to 170 mg. per 100 cc. of whole blood. The remarkable feature of Jacobsen's results is that he should have obtained clinically positive sugar in the urine and such high levels of glucose in the blood in so large a proportion of his subjects after the taking of only 100 gm. of glucose. It is perhaps worth noting that all of the subjects responding with positive alimentary glycosuria took the glucose 2 to 3 hours after breakfast, whereas the common experience indicates that the sugar tolerance is lowest before breakfast (Naunyn[9]). Quite recently, Hagedorn,[10] also from Den-

[7] Hamburger, H. J., and Brinkman, R., *Biochem. Z.*, 1918, lxxxviii, 97; 1919, xciv, 131; *Proc. K. Acad. Wetensch. Amsterdam*, 1918, xxi, 548. Hamburger, H. J., and Alons, C. L., *Biochem. Z.*, 1919, xciv, 129. Hamburger, H. J., *Proc. K. Akad. Wetensch.*, *Amsterdam*, 1919, xxii, 351, 360. *Brit. Med. J.*, 1919, 267.

[8] Jacobsen, A. T. B., *Biochem. Z.*, 1913, lvi, 471; Blodsukkerindholdet hos normale og ved Diabetes mellitus, Copenhagen, 1917.

[9] Naunyn, B., Der Diabetes melitus, Vienna, 1906.

[10] Hagedorn, *Acta med. Scandinav.*, 1921, liii, 672.

mark, seems likewise to have met with very little difficulty in obtaining alimentary glycosuria after giving from 63 to 120 gm. of glucose; only in Hagedorn's cases the sugar was taken before breakfast.

In commenting on Jacobsen's results Hamman and Hirschman[11] in their paper on the same subject suggest that the persons giving positive sugar tests for the urine must have had a subnormal tolerance for sugar. Yet this is scarcely a satisfactory explanation in view of the large proportion of positive results and in view of the fact that the blood sugar reached such high levels before the appearance of sugar in the urine. A more plausible explanation occurred to one of us (B.) who has had experience with Bang's drop method of blood analysis. The physical pain produced by repeated needle pricks in the fingers is distinctly greater than that experienced in having blood taken from a vein, and a certain amount of superimposed psychical hyperglycemia may have been sufficient to produce the positive results obtained. Jacobsen, it may be noted, obtained as high levels in the blood sugar by the giving of 160 gm. of bread as by giving of 100 gm. of glucose, whereas according to most other investigators starch feeding does not produce nearly so high a level of hyperglycemia.

The obstacle encountered in trying to determine the renal threshold for sugar in normal persons lies in the fact that the hyperglycemia *just* adequate for the production of glycosuria, if produced at all, lasts for so short a time that one very seldom succeeds in collecting samples of urine representing the desired period. It is, therefore, not surprising to find that the available valid data on this point are very few. The majority of the figures so far reported indicate that the glucose threshold for strictly normal persons most frequently lies between 160 and 180 mg. per 100 cc. of whole blood—and somewhat higher, about 190 mg., as the upper limit for the plasma. Threshold values lying above 180 for whole blood, or 190 for plasma, have not yet been found for normal persons.

The figures cited, 160 to 180 mg., are usually referred to as the normal glucose threshold level, but it would perhaps be more correct to call them the *highest* normal level for the glucose threshold, because lower levels are frequently obtained. Thus Hamman

[11] Hamman, L., and Hirschman, I. I., *Arch. Int. Med.*, 1917, xx, 763.

and Hirschman gave 100 gm. of glucose to each of six normal persons, and in none of these could they find a glucose level above 150 mg. per 100 cc. of whole blood (Lewis-Benedict method), yet two of the six showed sugar in the urine.   In several other persons not normal these authors obtained threshold values of 170 to 180 mg.; and by the subcutaneous injection of 0.6 to 1 mg. of epinephrine they succeeded, on the basis of an intake of only 65 gm. of glucose, in obtaining such gradual increases of the sugar level in the blood that accurate threshold values could be obtained in two cases. In these two cases the threshold was again found to be 170 to 180 mg.

Goto and Kuno[12] have recently published an extensive series of threshold experiments on 53 Japanese subjects using plasma instead of whole blood and employing Benedict's method (Myers-Bailey modification) for the sugar determination.   The sugar, 100 gm. of glucose + 250 cc. of water, was given on an empty stomach in the morning.   On the basis of a critical study of their essential data we would make the following summary of their results.   Twenty-two of the subjects showed sugar in the urine. In eight of these the sugar threshold (for plasma) was between 180 and 190 mg., in five between 160 and 172, and in eight the threshold fell between 128 and 155 mg.   In one case the value for the threshold fell as low as 120 mg., without there being any reason to suspect the existence of diabetes.

The exact records of the blood sugar level at which glucose begins to appear in the urine are far from numerous, yet it is clear that while fairly definite figures can be assigned to the upper limit of the sugar threshold the lower levels have practically no definite limitation, but gradually descend until one comes to the "cyclic glycosuria of the renal type" (Faber and Norgaard[13]) where there is a transient mild glycosuria after every meal, and finally to the chronic renal glycosuria, where the sugar threshold is so low that it is more or less difficult or even impossible to cause the level of the blood sugar to sink below the threshold.

There are two other important points to be noted in connection with the sugar threshold in normal persons, important because they emphasize the uncertainty which must necessarily be at-

[12] Goto and Kuno, *Arch. Int. Med.*, 1921, xxvii, 224.

[13] Faber, K., and Norgaard, A., *Acta med. Scandinav.*, 1920–21, liv, 289.

tached to any definite figure supposed to represent the threshold concentration. The first of these is the fact that the threshold determined on the basis of a descending curve of sugar concentration in the blood may be entirely different from the threshold derived from an ascending curve. Goto and Kuno, for example, report one experiment where the sugar elimination began at 150 mg. of glucose per 100 cc. of plasma, but continued until the plasma sugar sank to 60 mg.; and two others where the elimination began at about 168 mg. and continued until plasma levels of 77 and 99, respectively, were reached. The other important point is one recently elucidated and emphasized by Henriques and Ege.[14] The level of the blood sugar is not the same in venous and in arterial blood, particularly when there is hyperglycemia due to ingestion or injection of glucose. It is, therefore, precisely under conditions demanded for threshold determinations that such differences imply uncertainties as to the meaning of any exact figures given for the threshold. The differences are by no means uniform, but are quite insignificant when the level is distinctly below the threshold. That any such differences should be ascertainable shows how much more accurate are the modern methods for the determination of the blood sugar than were the methods by means of which the earlier investigators tried to prove the retention of sugar by the liver.

We do not feel justified in omitting from this discussion of the literature on the blood sugar threshold the contributions which have come from Ambard[15] and his pupils. These French investigators have introduced conclusions, explanations, and generalizations which will undoubtedly figure in the literature for some time to come—yet we have been unable to satisfy ourselves that they are based on sound experimental data or that the new concepts advanced represent much else than empirical, fanciful, and misleading speculations.

According to Ambard, the characteristic feature of the sugar threshold consists of its constant endeavor to adjust itself to the fluctuations in the blood sugar level, and the threshold itself is, therefore, constantly changing, up or down, each change being a

[14] Henriques, V., and Ege, R., *Biochem. Z.*, 1921, cxix, 121.

[15] Ambard, L., Physiologie normale et pathologique des reins, Paris, 2nd Edition, 1920. Chabanier, *Arch. d'Urol. de Necker*, 1919, ii, 1.

response to the stimulus of a change in the blood sugar concentration. In actual figures Chabanier thus finds that the threshold may correspond to any level between 107 and 520 mg. of glucose per 100 cc. of blood.

Ambard assumes that the "kidney secretion constant, 0.07," a constant derived from an empirically selected urea concentration in urine of 2.5 per cent, is equally applicable for all other substances which find their way into the urine by virtue of the secretory activity of the kidneys—provided that the concentrations of these substances in the urine are recalculated to concentrations which are isotonic with 2.5 per cent urea solution. It will be no small task to prove the validity of such a remarkable assumption.

In their attempts to explain the sugar elimination in terms of the Ambard hypothesis Chabanier and his associates endeavored to eliminate or annul the sugar threshold by means of increasing doses of phlorhizin until further increases of the phlorhizin produced no more increase in the sugar elimination. On the basis of this experimental condition they endeavored to determine whether the sugar threshold alone had been eliminated or whether at the same time there had been a general change in their so called secretory constant, 0.07. This constant had not changed for urea, and because of no change in the urea elimination they concluded that the secretory constant for glucose must also remain unaffected. It is not at all strange that a considerable degree of similarity should be found between the elimination of urea and the elimination of sugar in a completely diabetic animal to whom the sugar is a useless, indeed one might say, a foreign substance. Any other foreign innocuous water-soluble substance would probably behave in the same way. But to conclude from this that the reason for it all is a certain empirically selected "secretory constant" is fanciful speculation, for the kidneys are by no means the only organ involved in the retention or elimination even of foreign materials. The absorption, retention, and release of those substances by the tissues in general play an important part. But Ambard and Chabanier now make use of their secretory constant as if it represented an established fundamental fact, and proceed to calculate on the basis of it the sugar threshold prevailing in other glycosurias than that of complete phlorhizin diabetes. and the figures obtained for the threshold vary between 107 and

520 mg. In the light of the experimentally observed thresholds found by other investigators we consider these calculated threshold values as mathematical proof of the non-existence of the secretory constant. And indeed, to us at least, it seems infinitely more probable that the secretory constant, rather than that unknown something called the glucose threshold, should change normally merely in response to changing concentrations of sugar in the blood. It is at least important for the present to keep sharply apart all directly determined blood sugar threshold values and all so called threshold values obtained by calculation by means of Ambard's formula.

A review of the sugar threshold in diabetes does not come within the scope of this paper. While there may be a more or less general tendency toward a gradual increase of the blood sugar concentration at which glycosuria begins, it seems nevertheless to be a fact that many diabetics show the normal, and some even the subnormal, thresholds encountered in non-diabetic individuals (Faber). It should be important to determine, if possible, whether the high threshold values found in diabetes are an inevitable ultimate feature of the abnormal carbohydrate metabolism or an indirect result depending on a general deterioration of the kidneys. In connection with this problem it is suggestive that abnormally high blood sugar thresholds have been found in nephritis—associated with retention of nitrogenous waste products. In one such case Hamman and Hirschman found a threshold value of about 205, Mason[16] found a value of over 217, and Williams and Humphreys[17] obtained one threshold value at over 250 mg. (by Benedict's method) and two others where the value found exceeded 200 mg. The increased level of the sugar threshold is not the only abnormality of carbohydrate utilization encountered in these cases—the high blood sugar values so frequently occurring together with non-protein nitrogen values of 150 mg. or more indicate that some other factor than a decrease in the efficiency of kidneys is involved.

A clear understanding of the fluctuations of the blood sugar and the existence of a renal threshold for glucose would seem to be indispensable in any attempt to interpret such concepts as

[16] Mason, E. H., *Arch. Int. Med.*, 1918, xxi, 216.
[17] Williams, J. R., and Humphreys, E. M., *Arch. Int. Med.*, 1919, xxiii, 537.

the limits of sugar assimilation or that of carbohydrate tolerance, yet most of the literature on these two subjects is based merely on qualitative tests for sugar in the urine.

The term "carbohydrate tolerance" was introduced in connection with diabetes. It represented originally the amount of carbohydrate which a diabetic patient could take in the course of 24 hours without showing sugar in the 24 hour urine. In recent years the term "carbohydrate" in this connection has come to include the carbohydrates which are produced within the organism from protein materials. By the "limit of sugar assimilation" a term introduced by Hofmeister,[18] is understood the amount of sugar which can be taken in one dose without any demonstrable loss of sugar with the urine. This concept has also had clinical applications; in a number of diseases other than diabetes it was found that the limit of sugar assimilation was materially lower than in normal individuals. The test has usually been made in the form introduced by Bloch[19]—100 gm. of glucose taken after breakfast. The test was extensively used, particularly in Germany 20 to 30 years ago, but the results obtained were far from uniform either in normal persons, or in others, and the clinical value of the test has long been considered rather small.[20]

In its original form, and as generally understood, alimentary glycosuria implied the false assumption that the taking of a larger dose of sugar than just sufficient to produce some glycosuria meant that all the surplus sugar taken would pass into the urine. The erroneous character of this assumption was clearly pointed out, in 1895, by Linossier and Rogue,[21] who introduced the term "coefficient of utilization" to express the fact that the more sugar that is taken the more is retained and utilized by non-diabetic individuals. The same point was expressed by Allen[22] (1913) in the even more emphatic terms of his "paradoxical law of dextrose," according to which there is absolutely no limit ("except death") to the amount of sugar which the non-diabetic individual can utilize. The most exact data concerning the utilization of dex-

[18] Hofmeister, F., *Arch. exp. Path. u. Pharmacol.*, 1888–89, xxv, 240.

[19] Bloch, G., *Z. klin. Med.*, 1893, xxii, 525.

[20] Kraus, F., and Ludwig, H., *Wien. klin. Woch.*, 1891, iv, 855.

[21] Linossier, G., and Rogue, G., *Arch. med. exp.*, 1895, vii, 228.

[22] Allen, F. M., Glycosuria and diabetes, Cambridge, 1913.

trose have been furnished by Woodyatt[23] and his associates.   By continuous intravenous injection of glucose these investigators found that 0.85 gm. per kilo body weight per hour could be utilized without loss of sugar with the urine.   By continuous injection of twice as much sugar, 1.7 gm. per kilo per hour, about 10 per cent is lost with the urine; and with about four times as much, 3.6 gm. of glucose per kilo per hour, the loss is still only 35 to 40 per cent. Further increases in the amount of sugar injected are not accompanied by any increase in the percentage of sugar lost through the kidneys.

The rate of glucose absorption from the digestive tract, according to Woodyatt, never exceeds 1.8 gm. per kilo per hour, from which one would conclude that a transient loss of about 10 per cent of absorbed glucose is the maximum that could be obtained on taking glucose by mouth, and the rate of loss would necessarily be of very short duration even when very large amounts of dextrose, several hundred grams, are taken.

The retention of glucose by the non-diabetic individual might well be interpreted as a most striking illustration of the applicability of the mass law to metabolism processes.   But, as in most biological processes, all the factors involved are not clear or known. The renal threshold for blood sugar and the mass law cannot yet be combined into a harmonious whole as an explanation of the facts in the case.   According to the mass law alone there should be no sharp break anywhere in the relation between the amount of sugar absorbed and the amount excreted by the kidneys, yet in the renal threshold concept we have an expression of the fact that a very sharp break does occur at a certain point of blood sugar concentration.

Theoretically speaking it should be and is incorrect to divide urines, as clinicians always have done, into sugar urines and sugar-free urines, but there is no general agreement as to how much of an error or how important an error is represented by such a classification.   The most extensive investigations on this point yet published are undoubtedly those of S. R. Benedict and associates.

Before taking up the conclusions reached by Benedict concern-

[23] Woodyatt, R. T., Sansum, W. D., and Wilder, R. M., *J. Am. Med. Assn.*, 1915, lxv, 2067.   Woodyatt, R. T., *The Harvey Lectures*, 1915–16, xi, 326.

ing carbohydrate metabolism, particularly sugar retention *versus* losses of sugar, we venture to make the following summary of his experimental findings.

The basis for the work is the colorimetric determination of sugar in normal urines after all disturbing substances have been removed by the mercuric nitrate treatment of Patein and Dufau.[24] The sugar elimination, by 2 hour periods, under the influence of different diets was then determined. The subjects were two normal persons (assistants). The older person (O., age 51) eliminated on mixed diets 996 mg. per 24 hours; on a diet containing very little carbohydrates the sugar output sank to 775 mg., and in response to high carbohydrate diet the sugar output rose to 1,479 mg. The corresponding figures obtained from the younger subject were respectively 639, 500, and 1,528 mg. of sugar for 24 hours. These variations in sugar elimination did not immediately follow changes in the carbohydrate content of the food, as in the case in diabetic individuals. It took 3 days of high carbohydrate feeding, for example, to bring the excreted sugar up to 1,528 mg. in the younger person.

The urines were usually collected and analyzed, as stated, in 2 hour periods. The lowest values, 36 and 19 mg. per hour, were obtained between 6 and 8 in the morning, though equally low figures sometimes occurred between 3 and 7 in the afternoon. Every meal was followed by a marked temporary increase in the sugar output; but this increase was not materially dependent on the carbohydrate content of the meal, for the mixed diet occasionally produced a larger increase than the carbohydrate-rich diet.

The effects noted on giving definite amounts of carbohydrates in the form of sugar also merit attention. The giving of 40 gm. of glucose before breakfast was followed by an increase of the sugar elimination amounting to 50 mg. per hour (for 2 hours), while 60 gm. produced an increase of 254 mg. per hour in the case of the older subject. In the younger person 50 gm. of glucose had no effect, and 60 gm. produced an increase of only 7 to 13 mg. per hour. 20 gm. of glucose before breakfast had no effect on the older person, but the same together with the breakfast produced

[24] Patein and Dufau, *J. pharm. et. chim.*, 1902, xv, 221. Benedict, S. R., and Osterberg, E., *J. Biol. Chem.* 1918, xxxiv, 195.

an increase of 129 and 137 mg., respectively, above the increase obtained from the breakfast alone. 20 gm. of cane-sugar, whether with or without breakfast, had no effect on the sugar elimination. Similar but much smaller increases were obtained from the younger person. Finally on taking 25 gm. of glucose with each meal the 24 hour sugar elimination rose from 935 mg. to 2,350 mg. the 1st day and 2,190 mg. the 2nd day. At this point the authors felt that the sugar eliminations were reaching dangerous proportions, and they did not feel justified in continuing the experiment. Yet in the case of the younger person 25 gm. of pure glucose added to each meal produced no increase whatever in the 24 hour sugar elimination. In many of the experiments the sugar in the urine was determined before and after fermentation. The investigations did not include determinations of the blood sugar.

It is interesting to note that Benedict in his discussion of the work uses the term carbohydrate tolerance exactly as is done in writings on diabetes. In order to get away from the misleading clinical term glycosuria he introduces the term "glycuresis" to express the increase of the sugar elimination above that of the control periods.

The most important point that Benedict manifestly means to convey is that the quantitative increases in the sugar elimination, measured in milligrams and encountered in supposedly normal persons, is qualitatively or in origin of the same nature as is the clinical elimination of sugar in diabetes. In fact, he distinctly suggests that the difference between the normal and the diabetic individual may be wholly a quantitative one, just as is that between the mild and severe diabetic. And he suggests that if the total sugar elimination amounts to more than 1.5 gm. per day, the diet should be altered until this figure, or a lower one, is reached as the upper limit. As in frank diabetes so here with apparently normal subjects does Benedict lean toward the view that the cause of glycuresis is probably associated with some failure or deficiency or exhaustion on the part of the pancreas.

While Benedict's experimental data constitute a slender support for his rather startling conclusions, those data are interesting in that they furnish exact figures for the quantities of sugar occurring in the urines of normal persons and for the fluctuations in that sugar occurring at different times of the day and in response to the intake of food.

The experiments of Woodyatt and of Benedict represent opposite extremes with regard to the levels of sugar intake used as a basis for the study of the retention and the loss of glucose.

Before taking up the consideration of our own experimental material there is one other point in the preceding literature which needs to be briefly reviewed. It is generally acknowledged that for quantitative studies of the kidney function it is the concentration of a given substance in the plasma rather than in the whole blood which should give the more correct information. But the use of whole blood instead of plasma is so much simpler from an analytical standpoint as to be quite justifiable unless it can be shown that results so obtained are misleading.

Among the modern investigators on the sugar threshold only Goto and Kuno have used the plasma. At present the general concensus of opinion seems to be that the distribution of the blood sugar between the plasma and corpuscles is, on the whole, so nearly equal in the case of human blood that no serious error is involved in the use of whole blood for the analysis.

Quite recently, however, the correctness of this view has been flatly contradicted by Falta and Richter-Quittner,[25] and they have published a series of analyses which would seem to show that not only the sugar, but also the chlorides, and even the whole of the non-protein nitrogen, including the urea, occur exclusively in the plasma. The analytical findings are accompanied by far reaching theoretical explanations and deductions, but these can well be left out of consideration until the analytical data have been verified. The essential point in Falta's experimental work according to his own interpretation lies in the use of hirudin for the prevention of the clotting. Other anticlotting reagents, such as citrate or oxalate, are said to be useless, because they render the blood cells permeable to sugar, urea, etc., and thus hide the fact that in life the blood cells are quite impermeable to these products. By means of this explanation Falta and his coworkers have at once disposed of the contrary earlier results of other investigators, and have at the same time increased the difficulties of showing that their own results are not correct. It is very difficult at the pres-

[25] Falta, W., and Richter-Quittner, M., *Biochem. Z.*, 1919, c, 148; 1921, cxiv, 145.

ent time to obtain hirudin. Several Danish investigators,[26] Ege, Hagedorn, Gad-Andresen, and Warburg have, however, been in a position to repeat the work, have done so, and all find that the remarkable analytical results reported by Falta and his assistants cannot be verified in their laboratories.

For the present, therefore, the findings of the above mentioned Austrian investigators might be left without serious consideration, but the subject is important and we have in connection with our own work included the study of the distribution of sugar, amino-acids, urea, etc., between the plasma and the corpuscles.

Plan and Scope of Investigation.

Our own experiments with carbohydrates were begun for the purpose of determining the relationship which might be supposed to exist between the elimination of sugar and the concentration of sugar in the blood. In order to throw additional light on the character of the carbohydrates present in urine and blood we have determined the sugar both before and after hydrolysis. This procedure appeared to us particularly important when the ingested carbohydrate is more complex than glucose. Even when the ingested carbohydrate consists of a simple hexose the hydrolysis might furnish some supplementary information as to the mooted questions of how much of the blood sugar, or the sugar in non-diabetic urines, can be spoken of as glucose. The conflicting results obtained by means of fermentation tests have not proved particularly illuminating on this point, and it has seemed to us doubtful how dependable fermentation results can be in connection with sugars so small in amount as those present in the urine. Indeed, it seems to us doubtful whether the presence or absence of glucose in normal urine can be settled by chemical tests applied to the urine alone, and we hoped to solve that problem on the basis of indirect evidence.

In nearly all of our experiments the blood sugar has been determined in plasma as well as in whole blood, and was also determined both before and after hydrolysis. The volume per cent of the corpuscles was determined by the hematocrit method of

[26] Ege, R., *Biochem. Z.*, 1920, cvii, 246. Hagedorn, H. C., *Biochem. Z.*, cvii, 248. Gad-Andresen, K. L., *Biochem. Z.*, cvii, 250. Warburg, E. J., *Biochem. Z.*, cvii, 252.

Hedin. While theoretical objections can be raised against the absolute values obtained by this method, it certainly does give consistent figures. The sugar content of the corpuscles has been obtained by calculation. The change in the amount of sugar found in whole blood, plasma, and corpuscles after hydrolysis is shown in our tables by the plus or minus numbers accompanying the figures obtained before hydrolysis.

Much attention was given to the quick separation of some perfectly clear plasma. The blood was collected directly from the needle inserted in the cubital vein, in carefully paraffined centrifuge tubes, and the separation was finished in from 2 to 4 minutes after the tube was filled with the blood. 4 minutes may be given as the regular time, but this time was not used until we had thoroughly satisfied ourselves that it gave the same results as the shorter period. No oxalate or anything else, except the paraffin, was used to prevent clotting during the centrifuging. Clotting has at no time entered into the results, but minute amounts of potassium oxalate, about 15 mg. per 10 cc. of blood or plasma, were added before diluting with water. The "post mortem" diffusion between plasma and corpuscles, to which Falta ascribes positive findings for sugar in the corpuscles, is, we believe, absolutely excluded in our experiments. Within 15 to 20 seconds after the blood was drawn a part of it was beginning to rotate in a powerful centrifuge, and in less than 8 minutes both the whole blood and the plasma had been precipitated by the sodium tungstate method of Folin and Wu.[27] We, therefore, cannot entertain the slightest doubt about the fact that our analyses reveal the true distribution of the sugar between the plasma and the corpuscles.

No attempt was made to prescribe any uniform time interval for the passing of the urine, but the time of each urination was noted, and the amount of sugar and urine obtained was calculated as so much per hour.

[27] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81. For the precipitation of the plasma proteins we use only half a volume of 10 per cent sodium tungstate and of ⅔ normal sulfuric acid. Careful preliminary investigations had shown that these amounts precipitated all of the protein and gave filtrates suitable for all the determinations included in the system of blood analysis of Folin and Wu.

With reference to the analytical methods used, there is need for but little discussion. The blood sugar was determined by the method of Folin and Wu,[28] both before and after hydrolysis. For the hydrolysis 2 cc. of blood or plasma filtrate were acidified with 3 drops of 10 per cent hydrochloric acid, and the mixture (in the blood sugar tube) was heated in boiling water for 75 minutes. The added acid was then neutralized with 3 drops of a sodium hydroxide solution which was equivalent to the hydrochloric acid. The method used for the sugar determinations in urine is given in the preceding paper.

In experimental work of the kind involved here it was inevitable that many experiments should prove disappointing in the sense of not yielding results of the kind expected, or indeed any information of visible consequence. In the interest of space we have omitted many such; we are, in fact, giving only a selected series of results, but we have not omitted any experiments merely in order to diminish apparent contradictions.

Experiments with Glucose, Maltose, Dextrin, and Starch.

The marked increase in the sugar of normal urine which comes after every carbohydrate meal, and to which Benedict has given the name glycuresis, would seem to indicate that there is some glucose excretion below the blood sugar threshold. In order to discover the extent to which this is the case we have made a large number of feeding experiments with pure glucose. Pure sugars, particularly glucose, should be more effective than starches in raising the level of the blood sugar, and in our experiments with this sugar we hoped also to get further data on the threshold.

From the very beginning the results obtained have been quite different from what a careful perusal of the literature had led us to expect.

In Tables I to V are given the essential data of five typical experiments with not less than 200 gm. of glucose. The sugar was taken on a fasting stomach in the forenoon after a morning sample of the blood and urine had been obtained. The fluctuations of the sugar in the blood and urine were followed for several hours. The sugar of the urine is given in milligrams per hour, but the

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[28] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, xli, 367.

volume of the urine expressed in cubic centimeters per hour is also given so that the percentage of sugar can be calculated if desired. The percentage is, however, of little importance except in relation to the question as to whether the urines would give positive qualita-

TABLE I.*

Subject D-n.   Age 22 years.   Weight 75 kilos.   200 gm. glucose taken.
Result: Maximum subthreshold hyperglycemia but no glycuresis.

Time.	Blood.				Urine.		Remarks.
Feb. 25, 1921.	Sugar per 100 cc.			Cor-puscles.	Vol-ume per hour.	Sugar per hour.	Fasting.
	Whole blood.	Plasma.	Cor-puscles.				
a.m.	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*mg.*	
10.12							250 cc. water taken. Urine voided.
11.55	105+0	107−9	102+12	43			
p.m.							
12.00					50	21+3	
12.05							200 gm. glucose taken in 830 cc. water.
12.50	152+4	172−4	122+17	41			
1.30					111	18+2	
1.50	121−6	127−13	112+4	41			
2.30					35	23+4	
3.00	136−21	143−13	127−34	41			
4.00					29	23+5	
4.35	105−3	105−7	105+2	42			
5.30					27	21+2	
6.05	95+2	101−4	88+9				
6.50					76	22+4	
7.00							Dinner.
9.25					111	65+21	Note glycuresis after meal!

*Small, plus or minus, figures show increase or decrease after hydrolysis.

tive tests for sugar.   The sugar excretion per hour is quite independent of the volume of the urine.

5 to 7 hours after the ingestion of the glucose, each subject was allowed to eat an ordinary mixed evening meal.   A record of what he ate was kept, but these details were found to be of no impor-

tance and are therefore omitted. The tables are arranged in the order of the highest blood sugar found, the highest coming first.

From these tables it will be seen that the highest blood sugar level attained was 152 mg. per 100 cc. of whole blood and 172 mg.

TABLE II.*

Subject T. W. W-r. Age 24 years. Weight 69 kilos. 200 gm. glucose taken.

Result: Maximum subthreshold hyperglycemia but no glycuresis.

Time.	Blood.				Urine.		Remarks.
Mar. 8, 1921.	Sugar per 100 cc.			Cor-puscles.	Vol-ume per hour.	Sugar per hour.	Fasting. Urinated and took 200 cc. water at 9 a.m.
	Whole blood.	Plasma.	Cor-puscles.				
a.m.	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*mg.*	
10.30	93+11	96+3	89+22	41			
10.46					238	20+6	
10.50							Took 200 gm. glu-
11.25	138+4	166−24	100+42	43			cose in 600 cc. water.
11.46					100	33+4	
p m.							
12.20	90+15						
12.58					125	35+8	
1.30	54+9	58+4	47+19	38			
1.58					25	32+11	
3.35	100+2	106+6	90−4	38			
4.12					104	30+6	
5.10					142	23+5	5.10 Dinner.
7.05	92+2	102−12	78+22	40			Note glycuresis after meal!
7.17					366	43+6	
10.00					52	32+8	
a.m.							
6.00					73	32	March 9.

Small, plus or minus, figures show increase or decrease after hydrolysis.

in the plasma. We have ten other similar experiments with as many different normal persons (students), but the average of these is about the same as for the five given, and none reached as high a figure for the blood sugar as that shown in Table I.

In not a single normal case, therefore, have we been able to obtain a blood sugar high enough to give the alimentary glyco- suria which comes when the glucose threshold is reached.   Further discussion of this somewhat unexpected result will be given in a

TABLE III.*

Subject P. A. Ch-r.   Age 24 years.   Weight 60 kilos.   215 gm. glucose taken.

Result: Maximum subthreshold hyperglycemia but no glycuresis.

Time.	Blood.				Urine.		Remarks.
Mar., 1921.	Sugar per 100 cc.			Cor- puscles.	Vol- ume per hour.	Sugar per hour.	Fasting. Urinated and took 200 cc. water, 8.53 a.m.
	Whole blood.	Plasma.	Cor- puscles.				
a.m.	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*mg.*	
10.20	90+9	96+4	81+17				
10.29					66	22	
10.33							Took 215 gm. glu- cose in 500 cc. water.
11.20	138+14	154+3	116+28				
11.33					140	26	
p.m.							
12.17	94+2	110−5	72+12	43			
12.39					51	23	
1.45	87−5˙	100−0	70−12				
1.53					22	19	
4.45	85+5	92−8	77+21				
5.00					31	19	
5.15							Dinner.
7.40	125−2	132−14	116+14				
8.03					23	57	Note glycuresis after meal!
10.35					38	38	

* Small, plus or minus, figures show increase or decrease after hydrolysis.

subsequent section, but we would have the reader recall that A. E. Taylor[29] some years ago also met with uniform failure in attempting to produce alimentary glycosuria in normal persons (students) by feeding pure glucose.   Taylor evidently was not

[29] Taylor, A. E., and Hulton, F., *J. Biol. Chem.*, 1916, xxv, 173.

aware of the transient character of the hyperglycemia produced by sugar, for he examined only 24 hour urines.

The giving of 200 gm. of pure glucose not only failed to produce the glycosuria which accompanies the blood sugar threshold, but the tables show that the hyperglycemias which we did obtain are

TABLE IV.

Subject J. D. C-d. Age 22 years. Weight 61 kilos. 200 gm. glucose taken.

Result: Maximum subthreshold hyperglycemia but no glycuresis.

Time.	Blood.				Urine.		Remarks.
Mar. 11, 1921.	Sugar per 100 cc.			Cor-puscles.	Vol-ume per hour.	Sugar per hour.	Fasting. Urinated at 9.00 a.m. Took 200 cc. water.
	Whole blood.	Plasma	Cor-puscles.				
a.m.	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*mg.*	
10.35	90	94	85	43			
10.43					252	21	
10.45							Took 200 gm. glucose in 550 cc. water.
11.30	118	130	102	43			
11.40					61	21	
p.m.							
12.22	102	101	104	41			
1.15					98	26	
1.45	94	102	83	41			
2.50					52	20	
4.30	78	81	75	43			
5.10					27	17	
5.30							Dinner.
7.30	134	142	123				
7.40					25	33	
a.m.							
12.45					32	38	

wholly without effect on those lower levels of sugar excretion comprised in the term glycuresis. This is manifestly important. Since the sugar of normal urine is quite independent of the level of the blood sugar it must be considered exceedingly doubtful whether the sugar of normal urine is glucose, quite independently of whether it does or does not ferment. But if that sugar is not

glucose it is difficult or impossible to assume that it has any connection with such problems as carbohydrate tolerance, limits of glucose assimilation, or with any "diabetic tendency" or "prediabetic stage." The absence of any unmistakable glycuresis following the ingestion of 200 gm. of glucose is also important in that *it proves the concept comprised in the term glucose threshold to*

TABLE V.*

Subject H. B-d.   Age 34 years.   Weight 90 kilos.   200 gm. glucose taken.
Result: Maximum subthreshold hyperglycemia but no glycuresis.

Time.	Blood.				Urine.		Remarks.
	Sugar per 100 cc.			Cor-pus-cles.	Vol-ume per hour.	Sugar per hour.	
Jan. 16, 1921.	Whole blood.	Plasma.	Cor-puscles.				Fasting. Urinated at 9.05 a. m.
p.m.	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc*	*mg*	
1.25					39	20+7	
2.05	84+10	93+3	71+20	41			
3.00					30	17+2	
3.05							Took 200 gm. glucose in 1,000 cc. water
3.40	116+11	129−2	97+30	42			
4.00					44	19−4	
5.00	70+5	70+1	70+10	42			
5.30					80	23+0	
6.30	57−3						
7.00					30	21+5	
9.00							Protein-fat meal.
a.m.							
12.45					32	26+6	No glycuresis.
9.00					30	23+4	Following morning.

* Small, plus or minus, figures show increase or decrease after hydrolysis.

be not something only approximately true; the concept is absolutely correct, however uncertain the exact figures given for the threshold may be. Hyperglycemia definitely below the threshold does not normally produce the slightest leakage of glucose through the kidneys, and normally not a trace of absorbed and circulating glucose is lost.

The striking difference between pure glucose and ordinary mixed meals in their effects on the sugar excretion is shown in each of the

five experiments recorded in Tables I to V. The definite unmistakable glycuresis described by Benedict is obtained after every carbohydrate meal, but not after meals containing no carbohydrate (Table V).

By hydrolysis the sugar of the urine is increased. The sugar of urine is, therefore, made up, at least in part, of di- or polysaccharides, for we have satisfied ourselves that the increase after hydrolysis cannot be explained on the basis of glucuronic acids.

The conditions under which glycuresis is obtained and the increase after hydrolysis suggested the possibility that the sugar of normal urine is due to a little premature absorption of incompletely digested carbohydrate material, such as maltose and dextrins.

Two experiments were made with maltose, but only one is recorded here (Table VI). The maltose was a Kahlbaum preparation but was not absolutely pure. It contained enough dextrin to give a faint but unmistakable reaction with iodine. From the figures given in Table VI it will be seen that 200 gm. of maltose have had extremely little effect on the level of the blood sugar, and like glucose has no effect on the sugar of the urine.

The urine passed at 1.07 p.m., 62 minutes after the maltose ingestion, evidently contained a trace of "dextrin." It may seem rather extraordinary that any evidence of dextrin in the urine should be obtainable from the small amount of such polysaccharides present in the maltose, but the significance of the phenomenon is clear enough from the results which we have obtained from our feeding experiments with large quantities of dextrin.

Three experiments were made with dextrin with the subject H. B-d. The results obtained from these experiments seemed to furnish an adequate explanation of the origin of the sugar in normal urine and determined the trend of many subsequent experiments, but we discovered after a while that our original interpretation was erroneous, and the significance of the dextrin results was considerably diminished. We omit, therefore, one of the experiments in which 200 gm. of dextrin were taken.

Table VII gives the results obtained from 350 gm. of dextrin. The dextrin was taken in two doses about 2 hours apart. The subject suffered no discomfort from the large amount of dextrin taken and enjoyed his evening meal as well as the meals taken

during the subsequent days. There was no reason to doubt that the dextrin was digested and absorbed very much as an equivalent quantity of other carbohydrate food. From all this dextrin we obtained no increase in the level of the blood sugar and no increase

TABLE VI.*

Subject H. B-d. Age 34 years. Weight 90 kilos. 200 gm. maltose taken. *Result:* No glycuresis.

Time.	Blood.				Urine.		Remarks.
Nov. 9, 1921.	Sugar per 100 cc.			Cor-puscles.	Vol-ume per hour.	Sugar per hour.	Fasting. Urinated at 10.12 a.m.
	Whole blood.	Plasma.	Cor-puscles.				
a.m	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*mg.*	
11.45	90−0	90−4	90+5	41			
p.m.							
12.00					20	20+7	
12.05							Took 200 gm. maltose (Kahlbaum) in 600 cc. water.
12.30	80+4	108−6	36+20	39			
1.07					70	25+16	
1.20	78+7						
1.41					42	21+7	
2.22	69+8	73−0	63+20	38			
2.45					26	22+6	
4.05	75+7	78−5	70+26	39			
4.33					24	22+6	
6.30					21	19+4	Dinner at 6.45 p. m.
11.42					26	39+11	Note glycuresis after meal!
a.m.							
10.12					51	43+9	Second day.
p.m.							
12.04					24	22+2	Fasting.

* Small, plus or minus, figures show increase or decrease after hydrolysis.

of the preformed sugar of the urine. The urine contained, however, an abundance of dextrin. This was our second dextrin experiment, and its main purpose was to see how long the dextrin excretion continued, for we had found in the first experiment that the excretion was still rising at the end of 10 hours. And, as may be seen from the table, the dextrin excretion is at its height

in the morning of the day following the dextrin ingestion. The elimination of dextrin continued for 4 days. There can, therefore, scarcely be any doubt about the interpretation. Some dextrin or dextrin-like product had been absorbed from the diges-

TABLE VII.*

Illustrating the Slow Excretion of Absorbed Dextrin.

Subject H. B-d.   Age 34 years.   Weight 90 kilos.

Time.	Blood.			Corpuscles.	Urine.		Remarks.
	Sugar per 100 cc.				Volume per hour.	Sugar per hour.	
Jan. 22, 1921.	Whole blood.	Plasma.	Corpuscles.				Fasting. Drank 200 cc. water at 11.45 a.m. and again at 12.40 p.m.
p.m.	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*mg.*	
12.50					20	24+4	Preceding urine passed 9 a.m.
3.10	101+5	109−5	89+20	38			
3.35					24	23+2	Took 200 gm. dextrin in 800 cc. water.
4.18	101+0	100−3	102+4	41			
4.52					195	25+54	Marked diuresis.
4.55							Took 150 gm. dextrin in 500 cc. water.
5.20					460	28+137	Diuresis.
5.35	87−2	88+2	86−9	39			
5.50					580	34+170	Diuresis.
7.37					67	29+176	
7.50	83+2	95−1	66+7	41			
8.52					42	30+173	
a.m.							
9.00					30	35+185	Second day.
10.00					28	44+50	Third day.
9.00					32	38+28	Fourth day.

* Small, plus or minus, figures show increase or decrease of sugar after hydrolysis.

tive tract which after absorption behaved entirely like a foreign unusable product which slowly found its way into the urine. The absorbed material did not stay in the blood, for if it had been there our sugar determinations after hydrolysis would have revealed its presence. It must, therefore, have been absorbed by the tissues and was later gradually released and eliminated.

In order to be absolutely sure that the long continued dextrin excretion was not due to some unusual delay in the absorption from the intestine, another experiment was made with a single dose of 200 gm. of dextrin followed 2 hours afterwards by a large dose (3 tablespoons) of castor oil. The bowel was thus thoroughly cleaned out in the course of about 5 hours. The course of the dextrin elimination is shown in Table VIII.

TABLE VIII.*

Showing that Slow "Dextrin" Excretion Does Not Depend on Slow Absorption.

Subject H. B-d. Age 34 years. Weight 90 kilos.

Time.	Urine volume per hour.	Urine sugar per hour.	Remarks.
a.m.	*cc.*	*mg.*	
11.23	32	25+9	Fasting.
11.32			Took 200 gm. of dextrin in 600 cc. water.
p.m.			
12.18	48	27+51	
1.20	37	28+150	
1.25			Took 45 gm. of castor oil.
2.24	50	31+201	
3.44	179	40+250	
4.13	93	27+156	
5.01	34	24+133	
7.12	33	25+118	
a.m.			
12.52	28	22+75	Second day.
9.50	18	17+38	
11.25	15	17+24	Fasted throughout the experiment.

* Small plus figures show increase after hydrolysis.

The experiments with dextrin furnished the clue to the origin of sugar in normal urine, but our assumption that the dextrin excretion was due to the fact that we had overtaxed the digestive mechanism and that this was the reason for the absorption of unchanged dextrin was not correct. At least we do not now think that premature absorption of *digestible* dextrin has anything to do with it. We would recall in this connection the fact that we could detect the presence of some dextrin-like substance in one of the urines from the maltose experiment.

Our main reason for believing that the polysaccharide excretion after taking dextrin is due to the presence of some denatured, indigestible, and unusable carbohydrate rather than to the escape of real dextrin is based on the results obtained with pure starch. The commercial preparation of starch differs from the processes used in the preparation of dextrin and sugars in that practically

TABLE IX.*

Showing Absence of Glycuresis after 175 Gm. Pure Raw Potato Starch.
Subject H. B-d.　Age 34 years.　Weight 90 kilos.

Time.	Blood.				Urine.		Remarks.
	Sugar per 100 cc.			Cor-puscles.	Vol-ume per hour.	Sugar per hour.	
Nov. 26, 1921.	Whole blood.	Plasma.	Cor-puscles.				Fasting.　Urinated at 10.19 a.m.
a.m.	mg.	mg.	mg.	vol per cent	cc.	mg.	
11.20	93−4	98−13	85+10	39			
11.30					30	30+10	
11.55–							Took 175 gm. raw
12.25							potato starch in 600 cc. water.
p.m.							
1.00					130	25+9	
1.12	83−5	96−26	63+27	39			
1.23					625	33+14	
2.00	70+9	93−3	37+27	41			
2.22					58	21+7	
3.25	68+9	81−22	47+58	39			
3.57					33	21+10	
4.44	80+1	79−9	81+17	40			
6.52					28	20+9	

* Small, plus or minus, figures show increase or decrease after hydrolysis.

no heat and no vigorous chemical reagents are used in the manufacture of starch. There is thus good reason to believe that starch is free from decomposition products and free from soluble impurities of a carbohydrate nature.

Whether the carbohydrate elimination after taking dextrin is or is not due to impurities is of minor importance, for it is, after all, on the basis of pure starch and the sugars, maltose and dex-

trose, that one can definitely determine whether the normal sugar excretion after meals is a feature of our main carbohydrate metabolism or only a side issue due to the presence in our food of products which from the standpoint of carbohydrate metabolism can be regarded as impurities.

. Two experiments with starch are recorded in Tables IX and X. The starch (175 gm.) was first taken in a raw, uncooked, condition. 25 gm. of starch were first converted into a thin paste by means of hot water and into this was stirred the remaining starch (150 gm.). The mixture was eaten with a spoon and was very difficult to take.

TABLE X.*

Showing Absence of Glycuresis after 200 Gm. Pure Dextrin-Starch.
Subject H. B-d.   Age 34 years.   Weight 90 kilos.

Time.	Urine.		Remarks.
Nov. 28, 1921.	Volume per hour.	Sugar per hour.	Fasting. Urinated at 9.39 a.m.
a.m.	cc.	mg.	
10.15	33	23+11	
10.40–10.50			Took 200 gm. dextrin-starch in 900 cc. water.
11.30	100	25+11	
p.m.			
12.27	137	29+4	
1.40	37	24+10	
3.36	35	22+9	

* Small plus figures show increase after hydrolysis.

The analytical results given in Table IX show that from raw (potato) starch one does not obtain the glycuresis which is so prominent after ordinary mixed carbohydrate meals.   The blood sugar level did not rise appreciably, as was to be expected from the low levels which we have obtained from the sugars and from dextrin.

The experiment with raw starch was not deemed conclusive. While there was nothing to indicate that the starch was not completely digested and assimilated, the digestive process may have been so slow that the intermediate digestion products were immediately and completely converted into maltose and dextrose, thus eliminating the chance for any premature absorption of

intermediate products. We, accordingly, in the next experiment (Table X) first boiled 200 gm. of the starch with 900 cc. of water and then treated the paste at 70°C. with a little freshly prepared aqueous extract of malt. This diastase treatment was very mild, barely enough to soften the mixture so that it could be taken without the use of a spoon, but considerable dextrin was, of course, produced. This cooked and partially digested starch failed to produce glycuresis and also failed to yield any dextrin in the urine.

Before giving a more detailed discussion of the origin and character of the sugar of normal urine it is necessary to present some additional feeding experiments with sugars, for these have a more positive bearing on the problem than did the experiments with glucose and maltose.

Experiments with Fructose.

The feeding of fructose should give substantially the same results as those obtained with glucose, if it is true that fructose is converted into glucose in the liver and normally never gets beyond that organ. The assumption that fructose does not escape the liver is based on the fact that neither normal nor diabetic urines contain fructose even when large quantities of fructose or cane-sugar are taken. The fructose test for a much reduced efficiency on the part of the liver rests on the same assumption. The assimilation limit for fructose is also believed to be substantially the same as for glucose.

In Table XI are given the essential data on blood and urine obtained after taking 200 gm. of fructose. As far as the whole blood is concerned it will be observed that its sugar content has not increased. From the plasma sugar values we find, however, that there is here a slight increase (8 mg.), an increase which is hidden in the whole blood analysis by a nearly compensating decrease in the sugar of the corpuscles.

From the practically unchanged level of the blood sugar one should, of course, not expect to find any increase of the sugar in the urine, especially since none was obtained when the blood sugar did rise, as in the glucose experiments.

A material glycuresis lasting for several hours was nevertheless obtained, and the negative results obtained with Seliwanoff's

reaction showed that the sugar excreted could not be fructose. The urine passed at 5.43 p.m., 4 hours after the fructose ingestion, contained sugar equivalent to 0.25 per cent of hexose, and we could not have failed to get a positive reaction for levulose if even a small fraction of the sugar had been fructose. These data seemed to us beyond possibility of plausible explanation in terms of metabolism processes.

TABLE XI.*

Showing that 200 Gm. of Levulose Produces no Hyperglycemia and Does Give Rise to Some Unknown "Sugar" in the Urine.

Subject H. B-d.   Age 34 years.   Weight 90 kilos.

Time.	Blood.				Urine.		Remarks.
1921.	Sugar per 100 cc.			Cor-puscles	Vol-ume per hour.	Sugar per hour.	Fasting. Urine voided at 11.30 a.m.
	Whole blood.	Plasma.	Cor-puscles.				
p.m.	mg.	mg.	mg.	vol. per cent	cc.	mg.	
1.10	100	102	97	38			
1.30					27	26+6	
1.40							·Took 200 gm. levulose in 900 cc. water.
2.10	102	110	90	38			
2.40					63	40+1	
2.50	101	103	99	39			
4.05					36	42+2	
4.25	98	99	96	36			
5.43					22	57+0	
5.45	94	98	86	36			
8.10	93	95	90				
8.35					36	40+3	

* Small plus figures show increase after hydrolysis.

It occurred to us that the true explanation might be the presence of reducing decomposition products of levulose. The sugar used was a very high grade preparation of Kahlbaum's. Like all levulose preparations it had some color, and its concentrated solutions were yellow. Fructose is so unstable that it is very questionable whether any obtainable is entirely free from decomposition products. A very little of such reducing impurities (0.1

per cent) would be adequate to account for the sugar found in the urine. The prolonged character of the "sugar" excretion pointed distinctly to some foreign unusable impurity as the cause of the glycuresis.

Subsequent experiments made on this point have only served to confirm our conviction that we were here dealing with artificial products which have nothing to do with carbohydrate metabolism. How much such factors may have figured in all past feeding experiments with fructose one cannot tell. In the course of our investigation on the subject we heated 200 gm. of our pure levulose (dissolved in 300 cc. of water) at 145°C. for 1 hour. The levulose was so extensively destroyed that the sweet taste of this concentrated solution entirely disappeared. The resulting solution was considerably darker than before the heating, but by no means black. The solution was diluted to a volume of 500 cc. and its reducing power was determined. This was still equivalent to about 70 per cent of the levulose taken. In view of the unknown and doubtful character of this solution we hesitated about having anyone take the whole. Our fructose subject, H. B-d., took a small sample and observed no unpleasant symptom of any kind. A little diarrhea followed, but as a meal had been taken in between, it was not ascribed to the levulose decomposition products. The following day H. B-d. therefore took enough of the solution to correspond to about 150 gm. of the levulose. In about 1 hour a most violent diarrhea began and lasted for several hours. Considerable nausea was also experienced, but no headache or other intoxication symptoms. We deem it important to call attention to the vigorous laxative effects of decomposed levulose solutions. Maple sugar, molasses, and certain candies doubtless owe their laxative effects to similar decomposition products of sugar.

Because of the unexpected and dramatic outcome of this experiment we unfortunately neglected to pursue the original purpose for which the experiment was made as long as we should have done. Several consecutive samples of urine were, however, collected, and the analytical results obtained from these certainly confirm our original conjecture that the sugar in the fructose urines was derived from decomposition products of fructose. The sugar elimination, expressed in milligrams per hour, is shown in Table XII.

At a later time H. B-d. took 200 gm. of Schering's fructose. This sugar was in the form of a powder and looked white enough, but in solution it was much darker than similar solutions made from Kahlbaum's levulose. The diarrhea which followed the taking of this product was almost as intense as that obtained from the levulose which had been decomposed in the autoclave.

The main purpose of this experiment was to determine whether it is really true that fructose is completely retained and converted into glycogen in the liver. Since fructose has nowhere near the same effect as has glucose in raising the level of the blood sugar,

TABLE XII.*

Glycuresis after Decomposed Fructose.

Subject H. B-d. Age 34 years. Weight 90 kilos.

Time.	Urine volume per hour.	Urine sugar per hour.	Remarks.
a.m.	*cc.*	*mg.*	
10.45	33	28+9	Fasting.
10.45–11.10			150 gm. decomposed fructose in
p.m.			450 cc. water.
12.14	34	94+8	Diarrhea.
1.37	51	143+26	"
2.49	50	126+39	"
3.45	57	128+12	

* Small plus figures show increase after hydrolysis.

one is forced to assume that the transformation of fructose into glycogen within the liver is accomplished much more easily, and, therefore, more completely than is the manufacture of the same kind of glycogen from glucose. From the standpoint of chemical dynamics as well as from the standpoint of biological adaptation such a conclusion does not seem plausible. Levulose is fortunately a sugar for which we have a reasonably dependable and extremely sensitive test in Seliwanoff's reaction. By means of preliminary experiments we satisfied ourselves that we could detect levulose with perfect certainty in blood plasma if as much as 5 mg. per 100 cc. were present. The proteins of blood plasma can be precipitated by the sodium tungstate process without any preliminary dilution, and a filtrate can thus be obtained, the dilution of which is only double that of the original plasma. A

strictly fresh Seliwanoff's reagent containing 3 mg. of resorcinol per cc. of concentrated hydrochloric acid was used, and 2 cc. of this were added to 4 cc. of the blood filtrate—all for the purpose of eliminating avoidable dilutions. The blood for this test was taken 20 minutes after the ingestion of the levulose. The control blood was absolutely free from any substance giving the levulose reaction, and the test for levulose in the plasma representing a 20 minute absorption of levulose was unmistakably positive.

On the basis of these tests we affirm that levulose up to at least 5 mg. per 100 cc. of plasma may be present in blood at the height of active levulose absorption from the digestive tract. The blood sugar in the plasma was 98 mg. before and 112 mg. 20 minutes after the ingestion. At least one-third of the increase was, therefore, represented by levulose. The urine, as in other levulose experiments, contained enough sugar (reducing substances) to give positive clinical tests, yet we could not satisfy ourselves that any levulose was present. And a doubtful Seliwanoff's test applied to urine must necessarily be called negative.

Our own interpretation of the fate of absorbed fructose is as follows: The liver retains fructose as well as every other usable sugar to a greater extent in proportion to its weight than do the general tissues such as the muscles. But such retentions by the liver are never even approximately quantitative, and a large fraction of absorbed sugars, possibly the greater part, gets by this organ. Other tissues, such as the muscles, take up sugar from the plasma of arterial blood, and it is this general absorption which prevents excessive accumulations of sugar in the blood. But tissue sugar like blood sugar is normally and predominantly glucose, partly because the major part of our carbohydrate food is made up of glucose, partly because all other usable sugars are gradually converted into this essential sugar. The tissues being relatively well stored with glucose and empty of other sugars, such as fructose, may well be able to absorb these other sugars from the blood so nearly completely that the venous blood used for our analyses shows only traces. The glycogen formation may or may not begin immediately, and at all events need not be the immediate cause for the rapid disappearance of levulose from the blood. Levulose happens to be an excellent glycogen former, but that this is not the immediate cause of its disappearance from the

blood is strongly indicated by the results which we have obtained with a much poorer glycogen former—galactose.

Before taking up the discussion of our galactose and lactose experiments, we would call attention to one other important conclusion which we think can be drawn from our finding of levulose in the blood. Strictly speaking, there is only one substance for which there has been any reason to assume a renal threshold. The renal threshold for glucose is an abundantly established fact, and no substantial evidence for the existence of analogous thresholds for other materials has as yet been produced. Other alleged thresholds are based mostly on speculations. It would seem, however, that with reference to levulose we may have something corresponding to the threshold for glucose. Unless such a threshold for levulose came into play it is difficult to see why there should not have been levulose in the urine corresponding to the period of its proved presence in the blood plasma. The levulose should have been far more abundant than the reducing impurities which so readily found their way into the urine. The existence of a levulose threshold similar to that for glucose would serve to explain the occasional (though very rare) occurrence of fructosuria. The elimination of fructose in such cases would correspond to the elimination of glucose in renal glycosuria, and is caused by a lowering of the threshold for fructose. Fructosuria on the basis of this explanation would in most cases be a comparatively mild disorder, whereas without this explanation the elimination of fructose would have to mean a profound disturbance in the metabolism of carbohydrates, more or less analogous to diabetes mellitus.

Our facts and interpretations concerning the absorption and transportation of fructose suggest the possibility of making more extensive use of fructose in diabetes than has been possible in the past. It is impossible to tell how far past failures in the use of fructose may have been due to impurities, for they could doubtless bring about very serious upsets in the general condition of the patient. There is room for the suspicion that the early favorable impressions obtained as to the effects of fructose were based on the use of the purest obtainable brands, and that later, from economic considerations, cheaper brands loaded with impurities came into use. Continuous use of such would necessarily bring trouble.

The tissues of the diabetic person should contain much higher concentrations of glucose than tissues of normal persons, and this is the immediate reason why the blood sugar rises so high after the intake of glucose in any form. The high concentration of glucose in the tissues would probably have little or no effect on their absorption of fructose. From the giving of fructose we should, therefore, get a much higher concentration of total sugar (glucose plus fructose) in the tissues without any material increase in the sugar of the blood. Because of this higher concentration an increased utilization might well take place. Continuous or excessive use of fructose would, of course, defeat the purpose of the treatment, because gradually this would become equivalent to the loading of all the tissues with glucose, and, because of the large bulk of the tissues, an excessive amount of glucose would pour into the blood and be eliminated with the urine. Such a series of results has, however, no bearing on what would happen from small doses of pure fructose given at carefully regulated intervals. For under such conditions there is not only a larger concentration of sugar in the tissues in relation to the level of the blood sugar, but there is also the possibility that nascent glucose is more easily utilized than ordinary, preformed glucose. At least it seems safe to say that until the effects of pure fructose on diabetics have been carefully investigated from this standpoint the subject has not been exhausted.

Experiments with Galactose and Lactose.

All investigators who have made experiments with galactose are agreed that the assimilation limits for this sugar are entirely different from the assimilation limits for glucose and fructose. 20 to 40 gm. are usually given as the largest amounts that can be taken at once without resulting in glycosuria. The limits of assimilation found by any particular investigation would depend to a considerable extent on the analytical methods used in proving the presence or absence of glycosuria, or rather of galactosuria.

Our own investigations based on quantitative sugar determinations and planned to reveal the origin of the sugars found in normal urine have, of course, shown glycuresis at much lower levels of galactose (and lactose) ingestions than those based on qualita-

tive tests for sugar. Our data bearing on this point are given in Table XIII.

The finding of minute increases in the sugar of the urine after small doses of galactose has been to us a matter of minor significance in this particular case. It is the level of the blood sugar which from our point of view was a matter of fundamental importance. Galactose differs from levulose in that the former

TABLE XIII.*

Showing the Glycuresis, in Milligrams per Hour, Due to Galactose and Lactose.

All except the second experiment with 30 gm. galactose on Subject H. B-d. and that one on McC-n., the renal glycosuria subject.

Time after ingestion.	Pure galactose.				Pure lactose.				
	10 gm.	30 gm.	30 gm.	100 gm.	10 gm.	30 gm.	45 gm.	50 gm.	200 gm.
Before.	24+11	22	13	23+13	26+8	21+11	22+10	18+13	19+8
1 hour.	40+6	395	158	70+9	51+15		68+23	44+14	111+23
1½ hours.	43+9	153		538+46	33+10	53+20	80+31		252+148
2 "			284		29+12	42+21			
2½ "	22+8	23	37		23+8		40+16	63+24	
3 "			24	3,020−540		25+15		26+19	266+130
3½ "			17						
4 "								21+7	
5 "				452−12			27+14		175+99
7 "				33+20					76+61
8 "				25+17				21+9	

* Small, plus or minus, figures show increase or decrease after hydrolysis.

is assimilated very imperfectly, whereas the assimilation limits for the latter are so high that they can scarcely be exceeded. There is probably no difference of opinion about the cause of these widely separated assimilation limits. Fructose is on a par with glucose as a glycogen former; galactose is not. But if the glycogen formation in the liver were the chief factor by which the accumulation of absorbed sugar in the blood is prevented, then galactose should be immensely more effective even than glucose in raising the level of the sugar in the blood. 200 gm. of glucose will not yield a trace of sugar in the urine, whereas 100 gm. of galactose may yield as much as 10 gm. of excreted sugar.

Since a sugar excretion amounting to as much as 10 gm. has been obtained from 100 gm. of galactose it did not seem worth while to take any more, especially as we know from glucose experiments that 100 gm. produce just as high a hyperglycemia as does twice that quantity. The results obtained are given in Table XIV.

TABLE XIV.*

Showing that 100 Gm. Galactose Produces no Hyperglycemia but Does Give Rise to a Marked Galactosuria.

Subject H. B-d.　Age 34 years.　Weight 90 kilos.

Time.	Blood.				Urine.		Remarks.
	Sugar per 100 cc.			Cor-	Vol-	Sugar	
Dec., 1921.	Whole blood.	Plasma.	Cor- puscles.	puscles.	ume per hour.	per hour.	Fasting.　Urinated at 9.11 a.m.
p.m.	mg.	mg.	mg.	vol. per cent	cc.	mg.	
12.15	93	91	96	41			
12.24					33	23+13	
12.27				-			Took 100 gm. pure galactose in 300 cc. of water.
12.35	100	101	98	43			
12.52	89	89	89	42			
1.15					33	70+9	Qualitative sugar test positive.
1.23	87	88	86	42			
2.00					32	538+46	Qualitative sugar test positive.
3.28					64	3,020−540	Qualitative sugar test positive.
5.24					36	452−12	Qualitative sugar test positive.
6.50					31	33+20	After 6.50 p. m. free water intake.
8.11					119	25+17	

Total *extra* sugar excreted 5,685 mg.
* Small, plus or minus, figures show increase or decrease after hydrolysis.

The change in the level of the blood sugar is just about the same as that obtained from levulose. The transient maximum increase is 10 mg. per 100 cc. of plasma, yet in the course of 6 hours the sugar excretion amounted to 5.7 gm.

If to these data we add the observation that 10 gm. of galactose are sufficient to produce unmistakable glycuresis it is difficult to see how glycogen formation in the liver can possibly be the factor which prevented accumulation of sugar in the blood. On the basis of our explanation—absorption of sugars by the tissues—the observed facts are freed from inexplicable contradictions. Normally there is neither fructose nor galactose in the tissues, and within this enormous empty reservoir the galactose disappears as readily as does fructose and more readily than glucose. With respect to absorption and distribution galactose and fructose are alike. As far as excretion is concerned, the difference between the two is not even of a quantitative character. One is, the other is not excreted. Such a qualitative difference in the matter of excretion cannot very well depend on quantitative differences in the speed with which the two sugars are transformed into glycogen. The sugar excretion observed after very small doses of galactose clearly points to the absence of a renal threshold for galactose. In the absence of such a threshold galactose will continue to be excreted as long as there is any galactose in the tissues, for a little of it will constantly leak back into the blood. The excretion would be like that of absorbed dextrin except for the fact that even galactose is gradually converted into glycogen thus diminishing and finally exhausting the supply of galactose.

In a later section (page 270) we call attention to the diminution of the blood sugar obtained after hydrolysis. A similar diminution has in certain cases been found in the urine also. An extraordinary diminution was found in the galactose experiment. We have no explanation to offer, but are certain that the finding does not depend on analytical errors.

In connection with galactose we venture to make the same sort of a suggestion concerning its possible value in diabetes that we did for fructose (page 246). The peculiar possible merit of the galactose in this connection is that it is only very gradually converted into dextrose and glycogen. In this very slow transition we might have within the tissues a source for a relatively prolonged supply of nascent glucose. And if only 10 gm. were given at a time, the loss through the urine of unchanged galactose would be insignificant, notwithstanding the fact that it appears to be without a threshold.

Almost all authorities assign a much higher limit of assimilation for milk-sugar than for galactose, 100 to 150 gm. for the former, against 20 to 40 gm. for the latter. It is certainly true that much more sugar is excreted from 100 gm. of galactose than from 200 gm. of lactose, but with respect to the assimilation limits based on quantitative sugar determinations and glycuresis there is scarcely any difference between the two. 10 gm. of milk-sugar, corresponding approximately to the amount taken with a single glass of milk, is sufficient to produce a temporary, but unmistakable increase in the sugar of the urine.

The interesting fact about the sugar excretion after lactose ingestions is that *lactose* (as well as galactose) is present in the urine unless the amount taken is very small—less than 30 gm. This is instructive, for *it indicates that there is no mechanism in the digestive tract for preventing the absorption of soluble but incompletely digested carbohydrate materials. If such are not absorbed it is only because the activity of the endocellular enzymes is capable of accomplishing the hydrolysis during the transit of soluble carbohydrates through the mucous membrane of the intestine* In the case of lactose the endocellular hydrolytic activity is easily exceeded, and this disaccharide is absorbed. Lactose, as such, is supposed to be unusable, and it is customary to refer to the occasional occurrence of milk-sugar in the urine of pregnant women as proof of how completely unassimilable milk-sugar really is.

We do not think that the question of the assimilability of milk-sugar can yet be considered as definitely settled. The old experiments based on the injection of known quantities of milk-sugar into the blood and finding the whole of it in the urine can no longer be considered conclusive, because under such conditions the sugar in the urine would be increased by virtue of superimposed glycosuria. Our own experiments have not furnished conclusive evidence, but the fact that the excretion of milk-sugar comes to a definite end in the course of a few hours suggests that a part of the absorbed lactose may be utilized. The question of whether or not some lactose is utilized seemed to us unimportant in comparison with the problem of why milk-sugar taken by mouth results in less total sugar excretion than is obtained from the galactose corresponding to the same quantity of milk-sugar. 100 gm. of galactose will give twice as much sugar in the urine as 200

gm. of lactose. Our attempts to solve this problem have yielded extraordinarily interesting results, but our investigation is not yet completed, and the statements and interpretations here given we explicitly consider only as a preliminary communication. When the hydrolysis of the milk-sugar during absorption from the intestinal tract is quantitative, as it presumably is in normal infants,

TABLE XV.*

Showing that 200 Gm. Lactose Produces no Hyperglycemia and Does Give Rise to Lactosuria and Galactosuria.

Subject H. B-d. Age 34 years. Weight 90 kilos.

Time.	Blood.				Urine.		Remarks.
Feb. 6, 1921.	Sugar per 100 cc.			Cor-puscles.	Vol-ume per hour.	Sugar per hour.	Fasting. 200 cc. of water taken and urine voided at 9.30 a.m.
	Whole blood.	Plasma.	Cor-puscles.				
a.m.	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*mg.*	
11.15	94+4	96−8	90+24	38			
11.37					26	24+6	
11.40							Took 200 gm. lactose
p.m.							in 750 cc. water.
12.18	101+3	107−9	92+21	38			
1.00					135	160+52	
1.23	88+6	97+4	72+10	36			
2.00					56	290+46	
3.08	64+5	62−0	68+15	36			
4.00					29	255+58	
5.05	83+5	90−3	71+19				
5.50					21	108+34	Dinner at 6.30 p. m.
8.10	83+7	86+1	78+16				
9.00					25	66+34	
11.05					29	50+10	
a.m.							
11.00					34	21+5	Second day.

* Small, plus or minus, figures show increase or decrease after hydrolysis.

then the giving of milk-sugar is the same as the giving of equal quantities of galactose and glucose. The difference between the adult and the nursing infant with reference to the power of splitting lactose can be eliminated by giving pure glucose and pure galactose—equal amounts of each.

Table XV gives a picture of the sugar in blood and urine as obtained from 200 gm. of lactose. Table XVI gives the corresponding data obtained from 100 gm. of galactose plus 100 gm. of glucose. The sugar excretion is less than one-fifth as great from the mixture of the two sugars as from the milk-sugar and is less

TABLE XVI.

Showing that Galactose (100 Gm.) Is Almost Completely Retained if Ingested together with Glucose (100 Gm.).

Subject H. B-d. Age 34 years. Weight 90 kilos.

Time.	Blood.				Urine.		Remarks.
	Sugar per 100 cc.			Cor-puscles.	Vol-ume per hour.	Sugar per hour.	
Dec., 1921.	Whole blood.	Plasma.	Cor-puscles.				Fasting. Urinated at 9.45 a.m.
p.m.	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*mg.*	
12.00	94	97	90	41			
12.12					55	19	
12.15							Took 100 gm. glucose and 100 gm. galactose in 600 cc. water.
12.30	116	113	120	39			
12.48					244	35	
12.51	79	72	87	44			
1.05	79	84	72	43			
1.25					250	50	
1.35	82	85	78	42			
2.35					30	102	
4.13					30	137	After 4.13 p. m. free water intake.
5.08					224	71	
6.21					54	24	

Total *extra* sugar excreted 371 mg.

than one-tenth as great as the excretion obtained from 100 gm. of galactose when taken alone. The figures are 0.37, 2.8, and 5.7 gm., respectively.

The results given show that the extent to which galactose is retained and utilized by the human organism depends on the quantity of available glucose. The bearing of this finding on all past literature on the assimilation of galactose is clear.

The discovery is also interesting in that it seems to supply a simple and clear explanation of why milk-sugar should be advantageous for the young notwithstanding the fact that it has seemed to be the least useful sugar for adults. Galactose is needed, evidently in abundance, for the building of nerve tissue; and the surplus galactose is easily utilized, because of the presence of the glucose.

Possible practical applications of the knowledge thus acquired suggest themselves. Less milk-sugar and some pure glucose (or maltose) might be better than milk-sugar alone for infants whose rate of growth is subnormal or whose urine contains sugar. Galactose rather than milk might be advantageous for nursing mothers whose milk is low in lactose. One of us (H. B-d.) is particularly interested in these feeding problems and will report on them later.

Nature and Origin of the Sugar of Normal Urine.

From the experimental data given in the preceding sections there can be little or no room for doubt as to the origin of the sugars of normal urine, including the glycuresis discovered by S. R. Benedict. These sugars must consist of a considerable variety of reducing carbohydrates other than dextrose and fructose. A small portion may be derived from the sugar of milk. In the main they are derived from foreign, partly or wholly unusable, carbohydrate materials present in grains, vegetables, and fruits. Artificial decomposition products due to the heat used in cooking, canning, and baking probably contribute a considerable share. Baking and toasting should produce decomposition products similar to those encountered in dextrin. And wherever fructose or fructose compounds (cane-sugar, raffinose) are present, laxative decomposition products of fructose may be expected. All sweet preserves and many canned vegetables should contain such decomposition products. Pentoses and pentose compounds are doubtless represented, at least when fruit is eaten.

The escape of such miscellaneous unusable carbohydrate material into the urine has no connection with the main carbohydrate metabolism, and, therefore, none with the various problems of diabetes, except with reference to the correct interpretation of weak but positive tests for sugar in the urine. These foreign glycuresis products may sometimes be abundant enough to cause

confusion or to give positive clinical tests for sugar. It is clear that one cannot expect to find any sugar reagent which will entirely discriminate between such foreign sugars and traces of real glucose. Excessively sensitive copper reagents, such as the copper-glycerol reagent described by Folin[30] (1915), will show sugar in every urine.

TABLE XVII.

24 Hour Amounts (Milligrams) of Sugar in Normal Urine.

Subject H. B-d.   Age 34 years.   Weight 90 kilos.

Day.	Before hydrolysis.	Increase after hydrolysis.	Diet.		
1	980	738	Meat, eggs,	50 gm.	glucose.
2	611	159	" "	50 "	"
3	714	103	" "	50 "	"
4	756	73	" "	50 "	"
5	652	201	" "	50 "	"
6	543	101	" "	200 "	"
7	585	85	" "	50 "	"
8	624	52	" "	50 "	"
9	1,064	910	Mixed; including starches.		
10	1,360	770	"	"	"
11	1,090	200	"	"	"
12	1,650	485	"	"	"
13	1,170	450	"	"	"
14	885	815	"	"	"

While the absorption and excretion of foreign carbohydrates is to be sharply separated from the main carbohydrate metabolism, it does not necessarily follow that the precursors of the practically inescapable sugar of normal urine can be dismissed as of no importance. There is no reason to doubt that these products are absorbed from the blood by the tissues as readily as galactose and lactose and "dextrin." And the constant harboring of such foreign products in all the tissues of the body may very well be the cause of at least some minor disorders. As long as the etiology of so many skin and joint troubles remains unknown one cannot entirely dismiss the fact that the human organism is constantly charged with impurities of vegetable origin.

[30] Folin, O., *J. Biol. Chem.*, 1915, xxii, 327.

A survey of the various cooked and uncooked materials used as food can easily be made on the basis of feeding experiments accompanied by determination of the sugar of the urine before and after hydrolysis. Such a survey will show which products are particularly rich in soluble unassimilable carbohydrate materials. Fresh cider, for example (500 cc.), yields a little reducing sugar and considerable polysaccharides.

The sugars of normal urine cannot be entirely eliminated from the urine by abstaining from carbohydrate food, so that in addition to the precursors already referred to one must also ascribe a part to the endogenous metabolism. That this must be so is shown by the experiment continued for 14 days, recorded in Table XVII. During 8 days the subject, H. B-d., ate only meat, eggs, and butter, together with black tea and 50 gm. a day of pure glucose. The latter was added to exclude acidosis, because the fuel value of the food taken was probably not adequate for the daily energy requirements. The increase in the sugar excretion when the subject returned to ordinary mixed food was prompt and large.

On the Nature of the Glucose Threshold together with Some Observations on Renal Glycosuria.

The term alimentary glycosuria has been used to express the fact that some apparently normal persons eliminate moderate quantities of sugar after the ingestion of cane-sugar, glucose, or even after starch. The term alimentary glycosuria is a misnomer. The sugar is excreted either because the level of the blood sugar has risen above the normal threshold, or because the threshold itself is below the normal, as in renal glycosuria. There can be no doubt as to the existence of some mechanism by which the excretion of glucose is normally absolutely prevented. If we here venture to advance something approaching a new explanation we realize that it may be wholly wrong, but we believe that it should be at least partly correct. Each of us independently has in former researches seen and emphasized the avidity with which tissues may absorb materials from the blood.[31,32] And throughout this

[31] Folin, O., and Denis, W., Protein metabolism from the standpoint of blood and tissue analysis, *J. Biol. Chem.*, 1912, xi, 87.

[32] Berglund, H., Studier oever koksaltomsaettningens pysiologi och patologi, Stockholm, Norstedt, 1920.

investigation we have agreed that it is absorption by the tissues rather than glycogen formation which prevents excessive accumulations of the sugars in the blood. With reference to glucose we assume that the tissues always contain at least as high a concentration of free sugar as the blood plasma and probably more. The glycogen formation need not begin until the tissues have begun to possess a much higher concentration than that present in fasting. Much absorbed sugar can thus be distributed without any large increases of the sugar in the blood. But the kidneys receive their quota of sugar, just as do the other tissues, and this increase of sugar does not involve the slightest degree of strain. The strain comes only when the holding capacity for free sugar is reached and when the glycogen formation must come into play to keep the sugar concentration within normal limits. The speed of glycogen formation is of a much lower order than is the earlier process of merely absorbing the sugar from the blood. At this stage, therefore, the sugar backs up in the blood and *the holding capacity of some tissues including the kidneys is exceeded.* As a result of the strain thus produced the kidneys are finally compelled to make use of a more efficient process than the glycogen formation for reducing the sugar concentration in the kidney cells, and the elimination of sugar suddenly begins. That a real local strain has preceded the escape of the sugar is indicated by the fact that the sugar excretion once begun does not stop as soon as the blood sugar has fallen below the threshold, but, in fact, continues until the level of the blood sugar has gone away down, even to subfasting values (hypoglycemia). It is as if there had sprung a leak for sugar, a leak which cannot be immediately repaired. Yet the total amount of sugar eliminated need not be very large, except in clinical or experimental diabetes, because other tissues continue to absorb sugar, as well as to make glycogen.

We cannot here develop this interpretation in more detail because of its many ramifications. The essential point is that it makes clear why increasing concentrations of sugar in the blood below the threshold values involve no strain and no elimination of glucose. From our interpretation it also follows that excretion of glucose, as in emotional glycosuria and renal glycosuria, does not represent a finely adjusted normal process analogous to the excretion of sodium chloride or of waste products. It is also in harmony

with the belief in the importance of not permitting diabetic patients to excrete any sugar at all, if it possibly can be prevented.

From our interpretation of the renal threshold for glucose the study of renal glycosuria receives added importance. That inter-

TABLE XVIII.*

Showing that in Renal (as in Emotional) Glycosuria the Fall of the Blood Sugar Does not Immediately Stop the Excretion of Sugar.

Subject McC-n.   Age 22 years.   Weight 65 kilos.

Time.	Blood.				Urine.		Remarks.
	Sugar per 100 cc.			Cor-puscles.	Vol-ume per hour.	Sugar per hour.	
Apr. 1, 1921.	Whole blood.	Plasma.	Cor-puscles.				Fasting. Urinated at 8.56 a.m. and took 200 cc. water.
a.m.	mg.	mg.	mg.	vol. per cent	cc.	mg.	
10.15	100−2	98−4	101+2	45			
10.21					127	14	
10.25							Took 200 gm. glucose in 500 cc. water.
11.05	113+3	138−15	82+26	44			
11.21					145	526	
p.m.							
12.15	96+11	107+7	84+16	46			
12.21					97	365	
1.47	104−2	101+2	107−7	44			
1.58					159	97	
3.40	61−3	54−2	70−5	44			
3.48					178	34	
6.45					29	14	7.00 p. m. Dinner.
9.03	178−13	188−28	166+5				
9.08					48	200	
11.05					37	86	
a.m.							
7.05					41	20	Second day.

* Small, plus or minus, figures show increase or decrease after hydrolysis.

pretation raises, for example, the question whether other tissues than the kidneys are involved. Do these also have a smaller capacity for free sugar than normal tissues, or is this smaller capacity confined to the kidneys? We only raise the question, we are not now in a position to answer it.

Renal glycosuria is by no means uncommon; most observations on "alimentary glycosuria" represent nothing else. From a class of 100 students one can usually find at least one, and often two or more, who find sugar in their own urine when learning to make the tests for sugar. McC-n. was such a student. He found sugar in the urine after every meal, as well as after 50 gm. of cane-sugar, maltose, or glucose. In Table XVIII are given the figures for blood and urine obtained from this subject after the ingestion of 200 gm. of glucose. The highest observed level of the blood sugar was only 138 mg. per 100 cc. of plasma and 113 mg. per 100 cc. of whole blood. In less than 2 hours the blood sugar had fallen to 96 mg. per 100 cc. of whole blood. Such a typical fall in the level of the blood sugar may be taken as positively excluding diabetes—a view confirmed in the case of this subject by Dr. E. P. Joslin, who at the request of the young man's father (himself a physician) later examined him from the standpoint of possible diabetes. The sugar in the urine rose quickly to about 0.4 per cent and remained excessive for over 3 hours notwithstanding an extreme fall in the level of the blood sugar.

The evening meal following the glucose produced in this case an excessive rise in the level of the blood sugar, 178 mg. as against only 113 mg. obtained from 200 gm. of glucose.

It is a fairly well established fact that the effect on the blood sugar obtained from taking glucose is scarcely, if at all, dependent on the quantity· of sugar taken. The maximum level, coming 20 to 45 minutes after the ingestion, may be as great from 50 gm. as from 200 gm. Variable uncontrollable factors, which one must loosely refer to as the condition of the subject, play more of a rôle than the quantity of sugar taken. From Table XIX it will be seen that McC-n. gave a maximum blood sugar of 139 mg. from only 30 gm. of glucose, as against only 113 mg. previously obtained from the taking of 200 gm. of glucose. Some uncertainty is involved in this comparison because the maximum blood sugar level lasts only for a few minutes and is probably missed more often than found, but the conclusion that 30 gm. of glucose has in this case produced at least as high a blood sugar as did 200 gm. is justified by the figures obtained.

The table is given because it seems to show how small a quantity of glucose may produce a temporary glycosuria in cases of this

kind (renal glycosuria), and to emphasize how misleading it might be to interpret the results as indicating a "break down of the carbohydrate metabolism." When the blood sugar falls to the fasting level, or lower, in 2 to 3 hours after the taking of glucose, there is no reason to believe that the power to assimilate sugar is impaired.

TABLE XIX.

Showing that in Our Case of Renal Glycosuria Only 30 Gm. of Glucose Have Yielded Both Glycuresis and Glycosuria.

Subject McC-n.   Age 22 years.   Weight 65 kilos.

Time.	Blood.				Urine.		Remarks.
June, 1921.	Sugar per 100 cc.			Cor-puscles.	Vol-ume per hour.	Sugar per hour.	Fasting. Urinated at 9.23 a.m.
	Whole blood.	Plasma.	Cor-puscles.				
a.m.	*mg.*	*mg.*	*mg.*	*vol. per cent*	*cc.*	*mg.*	
11.29	97	102	92	44			
11.30					40	15	
11.31							Took 30 gm. glucose in 200 cc. water.
11.54	139	143	135	43			
11.55					48	27	
p.m.							
12.12	113	123	99	41			
12.13					57	94	Qualitative test positive.
12.36	75	77	72				
12.37					50	40	
1.08					45	14	

As a control on the results obtained with 30 gm. of glucose in a case of renal glycosuria, we give the experiment recorded in Table XX. The subject, D-n., had previously been used in an experiment with 200 gm. of glucose (Table I). In this case the sugar level in the blood rose only to 111 mg. and no glycuresis was obtained. D-n., who at this time was giving more or less assistance in the work, had accordingly become much interested, and had, of course, lost every trace of fear.

From our experiments with fructose and galactose we arrived at the conclusion that in the human organism there is a threshold for fructose, but none for galactose. On the basis of this interpretation McC-n. should not show any excessive (abnormal) excretion of galactose, and if he eliminated fructose it would indicate that the threshold for this sugar is intimately associated with the threshold for glucose.

TABLE XX.*

Control Experiment with 30 Gm. of Glucose (Compare Table XIX).

Subject D-n.   Age 22 years.   Weight 75 kilos.

Time.	Blood.				Urine.		Remarks.
June, 1921.	Sugar per 100 cc.			Cor- puscles.	Vol- ume per hour.	Sugar per hour.	Fasting. Urinated at 8 a.m.
	Whole blood.	Plasma.	Cor- puscles.				
a.m.	mg.	mg.	mg.	vol. per cent	cc.	mg.	
11.10	94−5	94−15	94+10	41			
11.12					28	17+3	
11.14							Took 30 gm. glucose in 300 cc. water.
11.30	93−14	94−16	91−11	40			
11.32					21	17+1	
11.47	111−17	114−23	106−6	40			
11.52					21	17+0	
p.m.							
12.05	101−15	103−17	97−10	39			
12.15					18	19+0	
12.28	97−19	102−21	90−17				
12.31					22	22+1	
12.52	89−12	92−22	86+2				
12.57					18	19+2	

* Small, plus or minus, figures show increase or decrease after hydrolysis.

The urine passed 1 hour after taking 50 gm. of our purest levulose (Kahlbaum's) gave with Seliwanoff's reagent exactly the same color as the urine passed immediately before the taking of the sugar. We are, therefore, certain that no fructose excretion took place. This negative result does not, of course, prove anything concerning the fructose threshold, because we do not know how much of it passed through the liver without being changed into

glucose. · The urine gave also negative results for glucose (qualitative tests), whereas positive qualitative tests were always obtained after 50 gm. of cane-sugar. It is, therefore, not probable that transformation into glucose was the reason why no fructose appeared in the urine. The quantitative determination gave scarcely any higher figures than would be accounted for by the impurities of the levulose.

The galactose experiment was made as follows: H. B-d., serving as control, and McC-n., the renal glycosuria subject, took on a fasting stomach 30 gm. of galactose in 200 cc. of water. In 3 hours the control subject eliminated 474 mg. of sugar after deducting the normal fasting amount (obtained by analysis of the urine passed before taking the galactose). The renal glycosuria subject eliminated only 404 mg. The lowered threshold for glucose is, therefore, without influence on the excretion of galactose, as should be the case on the basis of our conclusion that there is normally no threshold for galactose. The figures obtained are included in Table XIII.

On Emotional Hyperglycemia and Glycosuria.

One striking feature of all our experiments including those with glucose, maltose, dextrin, and starch, is that the blood sugar has not risen to the high levels which practically all other investigators have obtained from similar experiments. It is evident that these other investigators have introduced some factor favoring excessive hyperglycemia which we have accidentally avoided. We believe that this added factor is nothing else than the emotional state of the subjects. Our subjects, aside from H. B-d., were healthy medical students who offered to serve and who knew and learned from each other that no pain or danger was involved. It seems to us distinctly doubtful whether an equally favorable state of mind can often be obtained in the case of patients who stand in awe of the "doctor" and are unused to laboratory surroundings. We also believe that a relatively insignificant emotional disturbance has a greatly increased effect on the process of sugar mobilization when there is an active absorption of sugar from the digestive tract. It is quite true that those who have taken the blood from the fingers should get somewhat higher values for the blood sugar because if they prick deeply enough they will get uncontami-

nated arterial blood, but that this does not account for more than a part of the difference between their results and ours is shown by the fact that they so often get unmistakable glycosuria.

Cammidge, Forsyth, and Howard[33] have recently published a paper on the factors controlling the normal concentration of sugar in the blood, the results of which show, according to our interpretation, what misleading results may be obtained because of emotional sugar mobilization superimposed on the other experimental conditions. These authors took the blood from the roots of the finger nails. They have obtained substantially the same degree of hyperglycemia (140 to 180 mg.) from any kind of food except fat, as they obtained from sugar or other carbohydrates. They have accordingly reached the conclusion that the absorption of sugar from the digestive tract has nothing special to do with the obtained hyperglycemias.

Our experiments with egg white and with gelatin, dating from February, 1921, have yielded results entirely different from those reported by Cammidge, Forsyth, and Howard, and we are, therefore, forced to conclude that these authors were really dealing with emotional glycosuria. In one respect our results agree with theirs, namely in the fact that fat ingestion may lead to hypoglycemia—and it is in connection with hypoglycemia (next section, page 265) that we give the details of our findings.

Even under our conditions of working we' have not entirely escaped the superimposed effects of emotional glycosuria. One experiment illustrating the same deserves to be recorded (Table XXI). The subject, a medical student, S-g., never had any sugar in his urine (qualitative tests) either before or after the day of the experiment. When the preliminary sample of blood was taken he grew pale and very faint. When the second sample was taken he fainted completely. The plasma of this blood sample contained 210 mg. of sugar, whereas the first contained only 105 mg. In the course of the next hour the sugar content sank to 111 mg., and neither at this time nor later did the taking of the blood disturb him.

The figures for the sugar of the urine may be cited as one more illustration of the fact that when the glucose threshold is once

[33] Cammidge, P. J., Forsyth, J. A. C., and Howard, H. A. H., *Brit. Med. J.*, 1921, 586.

overcome by the rising tide of the blood sugar, the normal threshold is not restored for several hours.   In this case the excretion of glucose evidently continued till the plasma sugar had fallen below 80 mg.

TABLE XXI.*

Showing Effects of Glucose Ingestion Combined with Emotional Disturbance. Glycosuria Once Begun Does Not Stop at the Threshold.

Subject Mr. S-g.   Age 29 years.   Weight 74 kilos.

Time.	Blood.			Cor-pus-cles.	Urine.		Remarks.
Mar.29, 1921.	Sugar per 100 cc.				Vol-ume per hour.	Sugar per hour.	Fasting. Urinated at 9.10 a.m. and took 200 cc. water.
	Whole blood.	Plasma.	Cor-puscles.				
a.m.	mg.	mg.	mg.	vol. per cent	cc.	mg.	
10.15	90−1	105−18	69+25	40			
10.26					174	24−1	
10.40–10.48							Took 200 gm. glucose and 100 cc. casein digestion (4 gm. N) in 650 cc. water.
11.22	189−31	210−47	160−10	42			
11.40					55	1,260−310	
p.m.							
12.15	108−6.	111−20	105+12	42			
12.40					526	1,273−173	
1.37	88−5	81−8	97≠0	40			
1.41					162	43+7	
4.18	77−7	74−7	82−7	40			
4.26					60	22+8	
5.45	96−9	101−14	87+0				
6.00					21	20+6	
6.30							Dinner.
9.10					57	1,235−45	

* Small, plus or minus, figures indicate increase or decrease after hydrolysis.

In the evening, after dinner, the blood sugar must again have exceeded the threshold, for the urine passed at 9.10 p.m. contained over 2 per cent of sugar.   In this respect the result resembles that obtained from McC-n., the renal glycosuria subject.   We are under

the impression that excessive hyperglycemias are more easily obtained in the evenings than during the early forenoon hours, but have made no experiments bearing directly on this problem.

In order to prevent future misinterpretation of Table XXI we must specifically mention that S-g., like several other subjects, got several grams of mixed amino-acid nitrogen from casein together with the glucose. This variation was introduced in order to see whether nitrogenous materials could contribute anything to the raising of the sugar level in the blood, but as no such effect was found it has not seemed worth while to dwell upon this detail.

On Subfasting Blood Sugar Levels (Hypoglycemia).

In the preceding section we have referred to the important rôle which we ascribe to the mental state of a subject as a factor in the production of excessive hyperglycemias during active sugar absorption. Normally the general tissues are abundantly capable of taking up all the sugar which can be delivered by way of the intestinal absorption, but the tissue absorption from the blood may be interfered with by many factors which have no influence on absorption from the intestine.

The *rise* in the blood sugar has heretofore received a disproportionate share of attention. The fall of the blood sugar below the fasting values has been almost entirely neglected, yet such a fall we believe to be fully as normal and almost as regular a consequence of a carbohydrate meal as the rise. MacLean seems to be the only one who has given any special attention to this phase of carbohydrate transportation, or who has attempted to interpret the same.

The intial rise in the blood sugar is due, according to MacLean to the dormant glycogen-forming function of the liver, which does not begin to act until the blood sugar concentration begins to approach the threshold of 160 to 170 mg. The initial rise of the blood sugar is, therefore, due to this lag in the glycogen-forming process. Once started the glycogen-forming process gains momentum, overtakes and passes the speed of the sugar absorption from the intestine, thereby producing the subsequent fall in the blood sugar to concentrations far below fasting values. In the main this interpretation seems intelligible and plausible, except that it assumes a greater lack of sensitiveness or adjustibility, to varying

requirements, on the part of the sugar-storing mechanism than might be reasonably expected.   We find it difficult to suppose that the glycogen formation in the liver should not begin until the level of the sugar in the general circulation is approaching the threshold value.   The sugar concentration in the portal vein must

TABLE XXII.*

Showing that Protein (Raw Egg White) Does Not Change the Level of the Blood Sugar and Does Not Cause Glycuresis.

Subject H. B-d.   Age 34 years.   Weight 90 kilos.

Time.	Blood.				Urine.		Remarks.
Jan. 26, 1921.	Sugar per 100 cc.			Cor-pus-cles.	Vol-ume per hour.	Sugar per hour.	Fasting. Urinated at 9.15 a.m
	Whole blood.	Plasma.	Cor-puscles.				
p.m.	mg.	mg.	mg.	vol. per cent	cc.	mg	
12.55	108−3	114−7	99+3	40			
1.35					22	30+5	
2.15–							Took 1,000 gm. raw
2.25							egg white.
3.03	102−2	106−8	96+6	39			
3.25					115	27+7	
4.05	101−9						
4.35					282	28+6	
5.35	98−4	98−4	98−4	39			
6.05					71	26+5	
7.45	101−7	97−3	108−14	35			
8.35					58	24+6	
9.35	100−6	105−12	92+3	38			
10.25					49	21+4	

* Small, plus or minus, figures denote increase or decrease in sugar after hydrolysis.

reach this and higher values comparatively quickly, and the initiation of glycogen formation in the liver comes presumably from the sugar concentration in the portal blood.

MacLean and de Wesselow,[34] like most other investigators, have obtained materially higher levels of the blood sugar in response to various carbohydrate intakes than we get, and it must be in

[34] MacLean, H., and de Wesselow, O. L. V., *Quart. J. Med.*, 1921, xiv, 103.

part because of this fact that they assume that such levels are reached before the glycogen formation comes into active play to check the sugar accumulation.

The fall of the blood sugar, according to our interpretation, is not due to any unsuitable adjustment or excessive momentum of the glycogen-forming process. That fall comes when there is

TABLE XXIII.*

Showing that Protein (Gelatin) Does Not Raise the Level of the Blood Sugar.
Subject H. B-d. Age 34 years. Weight 90 kilos.

Time.	Blood.				Urine.		Remarks.
Feb. 7, 1921.	Sugar per 100 cc.			Cor-puscles.	Vol-ume per hour.	Sugar per hour.	Fasting. Urinated at 9 a.m.
	Whole blood.	Plasma.	Cor-puscles.				
p.m.	mg.	mg.	mg.	vol. per cent	cc.	mg.	
1.55	97−4	98−7	96+1	41			
2.25					27	33	
2.55–3.20							Took 135 gm. gelatin in 900 cc. hot water. No other food taken all day.
4.00	93−8	93+3	93−32	42			
4.37					39	38	
4.52	84+1	90−2	75+5	41			
6.03					39	44	
6.10	92−6	93−9	91−1	39			
7.15					77	70	
10.34					82	52	
10.55	98−7	102−5	92−10	39			
a.m.							
9.00					30	31	Second day.

* Small, plus or minus, figures show increase or decrease after hydrolysis.

reason to believe that all tissues are well supplied with available food material. It is an index of the fact that the need for sugar transportation from some tissues to others has temporarily fallen very low or ceased altogether. It is for this reason that the lowest levels and the longest periods of low levels are usually obtained after very large ingestions of sugars.

This interpretation is very elementary and perhaps incomplete, but it does agree with facts which cannot very well be explained on the basis of excessive glycogen formation, as, for example, the hypoglycemia obtained after the ingestion of gelatin and olive oil. Tables XXII, XXIII, and XXIV give the results obtained with 1,000 cc. of egg white, 135 gm. of gelatin, and 200 gm. of olive

TABLE XXIV.*

Showing Early Hypoglycemia after 200 Gm. Olive Oil.

Subject H. B-d. Age 34 years. Weight 90 kilos.

Time.	Blood.				Urine.		Remarks.
Feb. 19, 1921.	Sugar per 100 cc.			Cor-puscles.	Volume per hour.	Sugar per hour.	Fasting. Urinated at 11.40 a.m.
	Whole blood.	Plasma.	Cor-puscles.				
p.m.	mg.	mg.	mg.	vol. per cent	cc.	mg.	
2.15	96−10	100−8	89−13	37			
3.10					48	24+9	
3.15							Took 200 gm. olive oil and 250 cc. water.
4.20	70+1	72−8	68+12	41			
4.45					232	27+8	
5.53	88	95	77	40			
6.05					60	20+11	
7.30	92−1	93∓0	91−4	38			
8.07					39	22+4	
9.45	87−1	90−2	83+1	37			
10.17					42	22+5	

* Small, plus or minus, figures show increase or decrease after hydrolysis.

oil. The prompt and pronounced fall in the blood sugar (from 100 to 72 mg.) in the plasma after the olive oil is particularly remarkable, coming as it does within 1 hour after the ingestion.

The fall in the blood sugar after the gelatin is small, too small perhaps, to permit drawing any definite conclusion. The result is further obscured by the fact that it gave rise to a glycuresis. It is difficult to tell what impurities may be present in commercial gelatin preparations. At all events there is certainly no rise of the blood sugar either after gelatin or after egg white.

TABLE XXV.

Showing that Hypoglycemia Is No Less Frequent than Hyperglycemia after Glucose Ingestions.

Subject.	Glucose intake.	Whole blood-plasma sugar per 100 cc.			Remarks.
		Before.*	Highest.*	Lowest.*	
	gm.	*mg.*	*mg.*	*mg.*	
T. W. W-r. .	200	93–96	138–166	54–58	
McC-n.	200	100–98	113–138	61–54	
H. B-d	200	84–93	116–129	57	
L. A. C-n. .	200	86–96	132–143	66–61	Took 15 gm. urea with the sugar.
J. J. K-n.	200	95–105	168–203	66–74	Renal glycosuria and emotional hyper- glycemia.
Mr. P-r	150+150	89–92	109–113	67	2 hour intervals be- tween the sugar por- tions. Took 15 gm. urea with the first one.
Mr. S-g	200	90–105	189–210	77–74	Took 100 cc. casein digestion with the sugar. Emotional hyperglycemia.
McC-n	30	97–102	139–143	75–77	The lowest figure 1 hour 5 minutes after ingestion.
J. M. F-r	120	94–93	131–139	77–83	Took 250 cc. casein digestion (10 gm. N) with the sugar.
J. D. C-d. .	200	90–94	118–130	78-81	
P. A. Ch-r.	215	90–96	138–154	85–92	
D-n	30	94–94	111–114	89–92	The lowest figure 1 hour 38 minutes after ingestion.
D-n	200	105–107	152–172	95–101	

* The first number in this column represents whole blood, the second plasma.

In Tables XXV and XXVI are given summaries of the fluctua- tions encountered in the sugar of whole blood at various periods after taking different kinds of food. In explanation of the many extremely low minimum values found it should be stated that most

of the subjects availed themselves of our invitation to rest on a couch during the greater part of the day. This resting condition was unfortunately not imposed on all the subjects. It was necessarily omitted in the many experiments with H. B-d., one of the authors of this paper.

TABLE XXVI.

Showing Effects of Other Food than Glucose on Hyper- and Hypoglycemia.

Subject.	Food intake.	Whole blood-plasma sugar per 100 cc.			Remarks.
		Before.*	Highest.*	Lowest.*	
		mg.	*mg.*	*mg.*	
H. B-d....	200 gm. lactose.	94–96	101–107	64–62	
Mr. C-c-a.	200 " maltose.	88–93	116–122	67–71	
H. B-d....	175 " raw starch.	93–98	83–96	68–81	
H. B-d....	200 " maltose.	90–90	80–108	69–73	
H. B-d....	200 " olive oil.	96–100	No rise.	70–72	
H. B-d....	100 " galactose + 100 gm. glucose.	94–97	116–113	79–72	
H. B-d....	Mixed lunch.	95–90	No rise.	77–80	Fasting.
O. F-n....	" "	100–103	" "	86–83	"
H. B-d....	350 gm. dextrin.	101–109	" "	83–95	
H. B-d....	135 " gelatin.	97–98	" "	84–90	
H. B-d....	100 " galactose.	93–91	100–101	87–88	
H. B-d....	200 " fructose.	100–102	102–110	93–95	
H. B-d....	200 " dextrin.	93–96	115–110	98–94	Dextrin taken 3 hours after breakfast.
H. B-d.	1,000 gm. egg white.	108–114	No rise.	98–98	

* The first figure in each column represents whole blood, the second plasma.

Distribution and Character of the Blood Sugar.

From the many blood sugar figures recorded in this paper for whole blood and for plasma, there is, we believe, no escape from the conclusion that the corpuscles in the circulating blood are permeable to glucose. We are inclined to think that they are not only permeable, but that they actually can take up more sugar from the plasma than can be explained on the basis of diffusion

alone. We do not venture to stress the possible active absorption of sugar by the corpuscles and absorption analogous to that postulated for tissues in general; the conditions are too complicated to justify any dogmatic statement. But if we consider the sugar concentration in plasma and corpuscles in relation to the water content of each, we find that in every case the sugar concentration in the water of the corpuscles is greater, sometimes fully twice as great, as the sugar concentration in the water of the plasma. On the basis of 90 per cent of water in the plasma and 50 per cent in the corpuscles, a concentration of 90 mg. in each would correspond to concentrations of 100 and 180 mg., respectively.

Without making any allowance for different water contents we see from the tables that there is no very uniform correspondence between the sugar of the plasma and of the corpuscles. In the fasting condition (before breakfast in the morning) the sugar contents of blood and corpuscles are so nearly together that one gets very nearly the same sugar value on whole blood as on plasma. The latter tends to give slightly higher figures, but the differences are not such as to justify the demand that plasma must be used in clinical work for sugar determinations.

During periods of active sugar absorption accompanied by considerable increases in the sugar concentration there is a relatively greater increase in the plasma than in the corpuscles, but this difference is naturally of short duration because the hyperglycemia itself does not last very long. What the situation is in hyperglycemia of diabetes we do not know. The records in the literature bearing on this point can scarcely be accepted because no adequate precaution has been taken to separate the plasma under conditions precluding subsequent changes in the distribution of the sugar.

The lack of a decided uniformity in the relationship between the sugar of plasma and corpuscles is not to be wondered at. The incoming sugar can reach the corpuscles only by first passing through the plasma. In addition there are other factors to be considered. In the plasma there is no catabolism and no polymerization of sugar, whereas both these processes must be assumed to occur in the corpuscles—in the absence of binding evidence to the contrary. If we are justified in assuming that the sugar in reality is of a higher concentration in the water of the corpuscles

than in the water of the plasma, we have the added possibility that the corpuscles at times may lose sugar to the plasma.

From the sugar obtained after hydrolysis it has become clear to us that the subject under discussion, the distribution of sugar between plasma and corpuscles, is further obscured by the uncertainty as to the nature of a part of the blood sugar. There are present in the blood, and, we believe, also in the urine, reducing substances which disappear during the hydrolysis (heating on a water bath with 1 per cent hydrochloric acid). These substances are most prominent in the plasma, as is indicated by the fact that the plasma filtrates very frequently yield less "sugar" after than before hydrolysis. The whole blood filtrates may show either an increase or a decrease in the reducing value as a result of the hydrolysis. It is evident, therefore, that as far as the corpuscles are concerned, the predominant effect of hydrolysis is an increase of the sugar, and this increase is usually sufficient to more than counterbalance the decrease obtained from the plasma. As there is no reason to doubt the presence in the corpuscles of the same substances which in the plasma cause a diminution of the sugar, the increasing effects obtained from hydrolysis of the corpuscle extracts is probably in reality larger than it appears to be. The increases produced by hydrolysis derived chiefly from the corpuscles may be due wholly, or in part, to glycogen. This must perhaps be admitted though we have not been able to prove it. Oyster glycogen added to whole blood immediately before precipitating with sodium tungstate and sulfuric acid is precipitated so completely that not a trace goes into the filtrate.

It is conceivable that the glycogen of human blood corpuscles is a different glycogen, but it is perhaps more probable that the extra sugar obtained by our method is derived from some intermediate carbohydrate.

SUMMARY AND CONCLUSIONS.

1. In the absence of emotional complications or a subnormal renal threshold (renal glycosuria) the ingestion of pure glucose (up to 200 gm.), does not raise the level of the blood sugar above the threshold in normal persons, and no glycosuria is obtained.

2. Fructose, galactose, or lactose, as well as dextrin or starch, are much less effective than glucose in raising the level of the blood sugar.

3. Absorption of sugars from the blood by the tissues, rather than glycogen formation, is believed to be the immediate reason why the sugar fails to accumulate in the blood.

4. A renal threshold analogous to that for glucose is believed to exist for fructose, but not for galactose or lactose.

5. The retention and utilization of galactose depends on the amount of available glucose.

6. Hypoglycemia (subfasting blood sugar level) is probably quite as normal a consequence of carbohydrate ingestion as hyperglycemia, but comes later.

7. Hypoglycemia is probably a reflection or index of a decreased need for sugar transportation from one set of tissues to another. This condition is obtained when there is an abundance of available carbohydrate material in all the tissues. A general abundance of other suitable food than glucose, notably fat (olive oil) may, therefore, also produce hypoglycemia. Hypoglycemia (in venous blood) can occur even during a prolonged moderate sugar absorption from the intestine, because the absorbed sugar may get by the liver but does not get into the venous blood.

8. Definite "glycuresis" (Benedict) is obtained after every ordinary carbohydrate meal.

9. Glycuresis is independent of the level of the blood sugar, and is not normally obtained from the ingestion of pure glucose, maltose, dextrin, or starch. The transition from below to above the renal threshold appears to be so sharp and decisive that there is practically no intermediate stage represented by glycuresis.

10. Glycuresis represents the absorption and excretion of (1) foreign unusable carbohydrate materials present in grains, vegetables, and fruits, and (2) decomposition products due to cooking, canning, and baking of such food.

11. The sugar of normal urine consists, therefore, of a motley variety of carbohydrate products and carbohydrate derivatives including di- and polysaccharides.

12. The blood sugar is distributed in somewhat varying proportions between plasma and corpuscles. In fasting the distribution is nearly equal between the two.

13. The plasma sugar is usually diminished by hydrolysis, while the sugar of the corpuscles is usually increased. The corpuscles probably contain polysaccharides—possibly polysaccharides other than glycogen.

THE PREPARATION OF INULIN, WITH SPECIAL REFERENCE TO ARTICHOKE TUBERS AS A SOURCE.*

By J. J. WILLAMAN.

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From time to time the suggestion is brought forward that the tubers of the Jerusalem artichoke, or girasole, would be an excellent source of commercial fructose, either as a syrup or as crystals.[1] The advantages offered are the enormous acre yields of the tubers, and the greater sweetness of fructose over other sugars. Stein proposed to separate the inulin from the expressed juice, and then hydrolyze it to fructose. Cockerell's syrup was made by hydrolyzing the juice directly. Hudson suggests the separation of fructose as the calcium compound; this would be more economical when used in connection with the second method mentioned above. Other writers have not suggested a definite procedure. It thus appears that information is needed on the probable merits of the two methods. In making some preparations of inulin from girasoles, the writer had certain experiences and discovered certain facts that might prove of value in this connection, and also in connection with the preparation of pure inulin. They are recorded in the present paper.

Methods of Preparing Inulin.

There are three general methods of separating inulin from contaminating substances: (*a*) direct crystallization from water,

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[1] Stein, S., *Int. Sugar J.*, 1908, x, 218. Daniel, A., British Patent No. 109, 813, 1917; in *Chem. Abstr.*, 1918, xii, 156. Hudson, C. S., *J. Ind. and Eng. Chem.*, 1918, x, 176. Cockerell, T. D. A., *Mo. Bull. State Comm. Hort.*, Colorado, 1919, viii, No. 5. Willaman, J. J., *Science*, 1920, lii, 351.

(b) precipitation by 60 per cent alcohol, and (c) precipitation as the barium compound in excess of $Ba(OH)_2$, and subsequently decomposing with CO_2. All three methods involve a preliminary clarification of the juice, usually with lead acetate. Method (a) was first used by Kiliani,[2] and then by many other investigators with various modifications. Alcoholic precipitation was first used by Dragendorff,[3] and has frequently been used in connection with the third method, which was instituted by Tanret.[4] Dean[5] tested many modifications of these methods, and found that the best procedure is to clarify with lead acetate, add $Ba(OH)_2$ to excess, then methyl alcohol to 30 per cent. The Ba precipitate is decomposed with CO_2, and the filtrate evaporated to dryness. This material is then successively extracted with boiling 84, 70, and 60 per cent alcohol. The final residue is true inulin, and is purified by a further precipitation with 60 per cent alcohol. Irvine and Steele[6] used recrystallization from water, followed by prolonged washing in cold water, and dehydration with a series of alcohol and of ether solutions. The most essential object to be attained in any method is the separation of the so called true inulin from the inulides, or inuloids, such as the pseudoinulin, helianthenin, inulenin, and synanthrin of Tanret. These bodies form a more or less progressive series with inulin, differing indistinctly in solubilities and in specific rotations. They are all primarily anhydrides of fructose, although Tanret[7] believes that they are characterized by glucose components in different amounts. Thus, from the standpoint of preparing pure inulin, the problem is to segregate the latter from the inulides. But from the standpoint of preparing fructose from girasole tubers, the problem is to separate the whole group from the other constituents of the juice.

In experimenting with the preparation of inulin, various steps in the existing methods were subjected to scrutiny. Without

[2] Kiliani, H., *Ann. Chem. Pharm.*, 1880, ccv, 145.

[3] Dragendorff, Material zu einer Monographie des Inulins, St. Petersburg, 1870; quoted in Abderhalden, E., Handbuch der biochemischen Arbeitsmethoden, Berlin, 1911, ii, 185.

[4] Tanret, C., *Compt. rend. Acad.*, 1893, cxvi, 514.

[5] Dean, A. L., *Am. Chem. J.*, 1904, xxxii, 69.

[6] Irvine, J. C., and Steele, E. S., *J. Chem. Soc.*, 1920, cxvii, 1474. Irvine, J. C., and Soutar, C. W., *J. Chem. Soc.*, 1920, cxviii, 1489.

[7] Tanret, C., *Bull. Soc. chim.*, 1893, ix, 200, 227, 622.

giving all the details of the work, the points on which any concrete evidence was obtained will be briefly discussed.

Professor G. R. McDole, of the Idaho Agricultural Experiment Station, very kindly furnished the artichokes. The variety was not definitely known, but was probably *Helianthus tuberosus albus*. The tubers were worked up during the winter, some being used as late as February.

Extraction of the Juice.—Since inulin is extremely insoluble in water at freezing temperatures, it was thought that by freezing the tubers and expressing the juice immediately on thawing considerable impurities might be removed. Accordingly, a sack of tubers, 2,000 gm., was left outdoors for 24 hours at about –25°C., ground, thawed at 0–5°C., and pressed in a hand-press. 530 cc. of juice, of 1.14 specific gravity, were obtained. The residue was extracted with boiling water in the usual way, and both juice and extract were worked up for inulin. The extract gave a lighter colored preparation than usual, and of lower yield, indicating it to be somewhat purer inulin. The juice gave a crop of apparently good inulin; but when the mass was diluted with ice water and centrifuged (see below), the crystals almost completely dissolved.

A second lot of tubers was treated in the same way. The first crop of inulin from the extract gave $[\alpha]_D^{20} = -34.4°$, whereas that from the juice even after two crystallizations from water, gave $[\alpha]_D^{20} = -30.9°$.

Thus it is apparent that the above freezing procedure gives a better preparation of inulin, but that it is wasteful for the preparation of fructose from the total inulin bodies. Some further experience with the process is required before it can be unqualifiedly recommended.

Clarification of the Juice.—The usual procedure is to add lead acetate, filter, and delead with either hydrogen sulfide or a sulfate. It was found that the inulin bodies have great peptizing power; as soon as a slight excess of H_2S is present, colloidal lead sulfide is formed, and it is very difficult to destroy. Lead sulfate is often difficult to filter. Sodium carbonate and ammonium oxalate were tried as deleading agents, and the latter found to be superior to any of the others.

Daniel,[1] in his patent covering the manufacture of inulin, specifies clarification with alkali, using any one of seven common

hydroxides. Cockerell's syrup[8] was clarified with $Ca(OH)_2$, CO_2, and SO_2. Alkalinity with $Ca(OH)_2$ was tried on an extract of girasole tubers. A quantity of precipitate was formed, but it could not be filtered. After centrifuging, it was acidified with phosphoric acid, and again filtered. This gave a liquor almost devoid of color; in fact, it was the best decolorization obtained in any of the experiments. Other trials gave similar results. Crystallization of inulin was not as good as from a solution clarified with lead, there apparently being some gummy material present that impeded crystallization. Because of these results, lead acetate followed by ammonium oxalate, was always used in clarification. The lime and phosphate method is not good for the preparation of inulin, but it shows promise for a process involving hydrolysis of the clarified juice directly into sugar.

The Purification of Inulin.—The separation of inulin by means of its barium compound is a long process; at least it is much longer than crystallization from water or precipitation by alcohol. It was decided to make a study of the relative merits of the latter two methods, comparing the yields and quality of products obtained by crystallizing from an aqueous syrup, and by precipitating from a medium containing 60 per cent of alcohol, this being the concentration generally agreed upon by workers as differentiating inulin from its congeners.

Two portions of a sample of inulin, $[\alpha]_D^{20} = -34.4°$, were dissolved in water to a 12.5 per cent strength. One was allowed to crystallize over night at 0–5°C., and was then filtered, washed with ice water, and then with 20, 50, 80, and 95 per cent alcohol, and finally with ether. It was then dried in an oven at 95–100°C. The other solution was made to a 60 per cent alcohol content and let stand over night. It was then filtered, washed with 60, 80, and 95 per cent alcohol and ether, and dried. The following data were obtained:

Crystallization from:	Yield.	$[\alpha]_D^{20}$
	per cent	
Water	80	−37.1°
60 per cent alcohol	94	−36.5°

[8] Personal communication.

The data in Table I were selected from the results of several experiments, which were not designed primarily to show the solubility of inulin in alcohol.

Table I shows that inulin has an appreciable solubility in 60 per cent alcohol, and that hence the yield of inulin from alcoholic precipitation is greater the more concentrated the solution of inulin that is used.

A more thorough comparison of crystallization in water and in alcohol was next made. A composite sample of inulin, made up of many small lots, and totaling about 100 gm., was washed by grinding in a ball-mill with 65 per cent alcohol, filtering, dissolving in boiling water, filtering with kieselguhr, and evaporating to a thin syrup (about 1.13 specific gravity). This was divided into

TABLE I.

$[\alpha]_D^{20}$ of the preparation used.	Concentration of inulin in the solution precipitated by 60 per cent alcohol.	Recovery of inulin.	Concentration of inulin in the 60 per cent alcohol filtrate.
	per cent	*per cent*	*per cent ·*
$-34.4°$	2.0	38	0.46
$-34.4°$	2.5	59	0.40
$-36.5°$	12.5	94	0.13
$-36.3°$	25 to 27		0.50
$-31.4°$	25 to 27		0.35
$-33.2°$	25 to 27		0.51
$-33.3°$	25 to 27		0.64

two portions. One portion was used for successive crystallizations from water, the other for successive crystallizations (precipitations) from 60 per cent alcohol. The one for the aqueous process was kept at 0–5°C. for 24 hours, at which time it had formed a solid mass of crystals. It was stirred with an equal volume of ice water, filtered, washed with ice water, then with 20, 50, 80, and 95 per cent alcohol and then ether. It was dried to constant weight at 100°C., and its specific rotation determined. It was then redissolved in hot water, filtered with washing, concentrated to a content of 25 to 27 per cent inulin, and crystallized as before. The filtrate and washings from each of these lots of crystals were concentrated to a content of 12 to 16 per cent inulin, made to 60 per cent alcohol by means of 95 per cent, and then treated in the same way as the other crystallizations from alcohol.

That portion of the original inulin syrup designed for successive precipitations by alcohol, after standing 24 hours at 0–5°C., was filtered cold, washed with cold 60, 80, and 95 per cent alcohol and then with ether, and dried as in the case of the aqueous samples. Each sample was redissolved in hot water, filtered with washing,
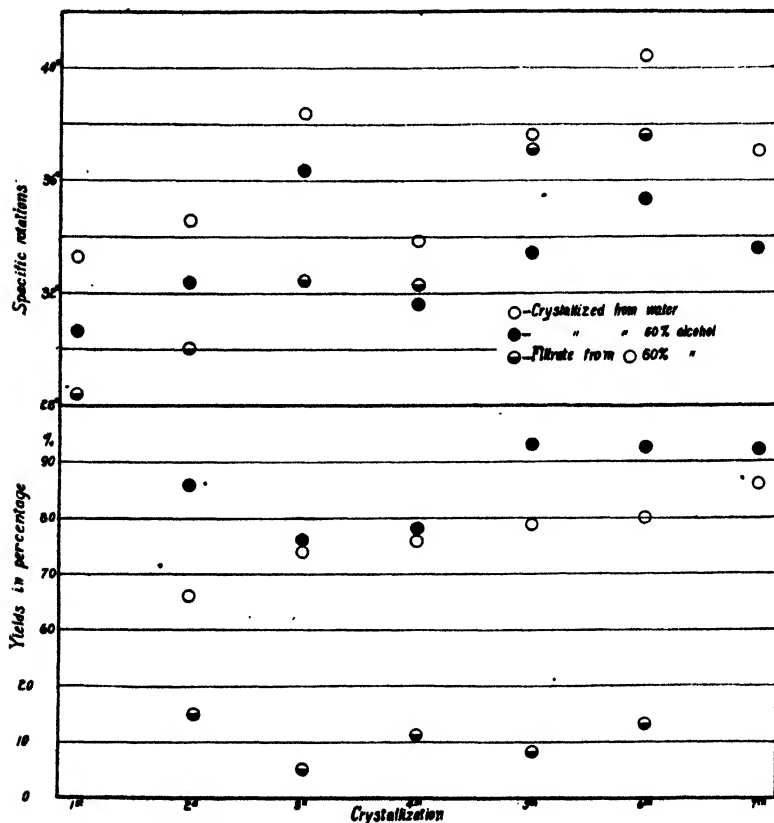
FIG. 1. Chart showing the specific rotation and yield of crystals of inulin prepared by successive crystallizations from water and from 60 per cent alcohol.

concentrated to a content of 25 to 27 per cent inulin, and again made to 60 per cent alcohol.

Fig. 1 shows the results of these successive attempts at purification. Both the specific rotation and the yield are given in each case.

It will be seen that the rotations of both the aqueous and the alcoholic samples increase during three recrystallizations, then decrease markedly on the fourth, then increase to a maximum, and finally undergo another decrease. The filtrate from the aqueous preparations does not show this phenomenon, but exhibits an almost continuous increase in rotation. It should be remembered that these latter preparations represent the inulin which is left in solution in the mother liquor, and which is precipitable by 60 per cent alcohol. They always show a considerably lower rotation than the parent samples, but this difference becomes less and less during successive recrystallizations. Unfortunately the last sample was lost; and the recrystallization had to be discontinued at the seventh one because the amount of material had become too small to work with. Dean reports the same experience in trying to purify inulin to a constant rotation. When his preparations, crystallized from water, reached values of 38 to 40, a further crystallizing always brought about a reduction in this value. In the present work, the inulin from the aqueous medium always had a higher rotation than the corresponding preparation from alcohol. The yields were higher from alcohol, however.

The above data constitute rather convincing evidence that inulin is not a single substance, but a mixture; and a somewhat unstable mixture at that. The successive crystallizations amounted to a fractionation, with the leaving behind in the mother liquor of those molecules of lesser specific rotation. When 60 per cent alcohol is the medium, the separation is less marked, due probably to the fact that the differential solubility of the two groups is greater in water than in alcohol. This is further shown by the greater yields from alcohol. Dean came to this same conclusion as regards the identity of inulin. He says,

"May we not, in a purely empirical way, call the carbohydrate, or carbohydrate mixture, which is readily precipitated in cold alcohol of 60 per cent strength, and has a specific rotation of $[\alpha]_D = -33°$ to $-40°$ inulin; and the undetermined mixture of lower rotatory power and greater solubility, the levulin mixture?"

He postulates the existence of various aggregates of molecules, more or less loosely combined; the greater the aggregate, the

greater is its specific rotation, and the lower is its solubility in water. This theory explains fairly well the behavior of inulin on successive crystallizations, either from water or from alcohol.

The Girasole as a Source of Inulin.—Tanret reports an analysis of the extract of girasole tubers which showed that of the total carbohydrates, inulin was 20 per cent; pseudoinulin, 0.3; inulenin, 9; helianthenin, 6; synanthrin, 50; sucrose, 12; and glucose and fructose, 3. This would indicate that these tubers are a poor source of true inulin. The writer's experience has shown this to be the case; the inulides greatly predominate over the inulin in all the lots of tubers examined. After precipitating a given extract with 60 per cent alcohol, no appreciable further precipitate was obtained until the concentration of alcohol reached about 84 per cent, when a voluminous coagulum was obtained. On the average, the yield of inulin of specific rotation of $-33°$ or over was about 2 to 5 per cent of the weight of the tubers, whereas the total inulin bodies must amount to three or four times this. The records in the literature go to show that dahlia tubers are a better source of true inulin.

CONCLUSIONS.

1. On the basis of the experience outlined in this paper, the writer recommends the following procedure for the preparation of inulin from artichoke tubers: Grind the washed tubers as fine as possible, and put into boiling water containing calcium carbonate. For each kilo of tubers use 1,300 cc. of water and 30 gm. $CaCO_3$. Boil 15 to 20 minutes, extract the juice with a press, reboil with 1,000 cc. of water and 10 gm. $CaCO_3$, extract, and combine the extracts. Clarify with lead acetate, avoiding a large excess. Centrifuge, or filter, remove the lead with ammonium oxalate, and centrifuge again. The clear liquor may here be treated with decolorizing carbon, although this is usually not necessary. It is then evaporated under vacuum to a content of 40 to 60 per cent of solids. This syrup is allowed to cool slowly, then it is kept at 0–5°C. for several hours, thoroughly stirred with an equal volume of ice water, and centrifuged. The crystals are redissolved in about 3 volumes of water, filtered hot, concentrated to about twice the volume of the original crystals, and allowed to crystallize in the cold as before. They are again

stirred with ice water, filtered on paper or on silk bolting cloth with suction, keeping everything as cold as possible. The crystals are washed with cold water, then with 20, 50, 80, and 95 per cent alcohol and ether, and dried in an oven at 100°C. The specific rotation of the preparation should now be at least −33°. A third crystallization may be performed, although it is useless to attempt to obtain a higher rotation than −38° or −39°. The washing method of purification of Irvine and Steele[6] may also be used, although the writer has not had experience with it.

2. Girasole tubers are not a satisfactory material for the preparation of true inulin, but are admirably adapted for investigations of the whole group of inulin substances.

3. For the manufacture of a fructose syrup from these tubers, a preliminary separation of the inulin bodies and their subsequent hydrolysis to fructose is not feasible, because only a small proportion of the inulin bodies present will crystallize from water. In all probability the best method for making a syrup will be along the lines of the following procedure: (a) the extraction of the juice by diffusion; (b) the clarification of the juice by means of lime, phosphoric acid, and decolorizing carbon; (c) the acid hydrolysis of all the inulin bodies; (d) the separation of fructose as calcium fructosate; and (e) the decomposition of the calcium fructosate and evaporation of the fructose to a syrup.

4. Dean's hypothesis that inulin is a group of substances with large, loosely bound molecules, and not a single substance, is supported by the evidence in the present paper.

THE UNSATURATED FATTY ACIDS OF LIVER LECITHIN.

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In previous communications on liver lecithin results were reported showing that the unsaturated fatty acids obtained on hydrogenation gave stearic and arachidic acids. It was further reported that the iodine number of the mixed unsaturated fatty acids was 154, and that the mixed unsaturated acids on bromination formed a bromide, which on the basis of its bromine content and of its melting point seemed to be the octobromarachidic acid.

From these findings it was concluded that liver lecithin contained more than one unsaturated fatty acid, and that the individual acids differed in the degree of their unsaturation. It then seemed suggestive that the acid of higher unsaturation was the tetra unsaturated arachidic acid. Since there was no accurate knowledge either as to the number of the unsaturated acids present in the mixture or as to the degree of their unsaturation, there was no indication as to the ratio of the individual acids. In order to complete our knowledge of the unsaturated fatty acids, it became necessary to find: (1) the number of the unsaturated fatty acids present in the liver lecithin; (2) the exact nature of the acids of higher unsaturation; and (3) the ratio of the individual fatty acids.

In the course of the present work it was found that liver lecithin yields only two unsaturated fatty acids, oleic and arachidonic[1] acids, and of these oleic acid predominates.

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[1] Since the tetra unsaturated arachidic acid is called *arachidonic* acid by Lewkowitsch (Lewkowitsch, J., Chemical technology and analysis of oils, fats and waxes, London, 4th edition, 1909, i, 211) this name will be adopted by the present writers.

In connection with the finding of arachidonic acid as a constituent of liver lecithin, it is interesting to recall the work of Hartley,[2] done under the direction of Leathes. On oxidation of the unsaturated acids from liver fat, including phosphatides, this author isolated an unsaturated fatty acid which was oxidized to a tetrahydroxyarachidic acid. Furthermore, on bromination of the same fatty acids Hartley obtained a bromide, having a bromine content only 0.7 per cent higher than that required by theory for octobromarachidic acid.

The present writers,[3] and Levene and Ingvaldsen,[4] have previously reported that on hydrogenation of the unsaturated acids of liver lecithin, arachidic acid was isolated. They have also obtained from liver lecithin a bromide, which on analysis gave a bromine value about 1 per cent lower than that required by theory for the octobromarachidic acid. In the course of the present work an octobromarachidic acid was isolated which gave correct analytical values. This acid was then reconverted into a tetra unsaturated arachidic acid, which on hydrogenation was converted into arachidic acid. From the product of bromination of the unsaturated fatty acids, fractions were obtained which analytically resembled the hexa- and tetrabromides. On purification of these fractions only the octobromide was isolated in pure form. Hence present day evidence does not permit us to assume the presence of more than one poly unsaturated fatty acid in lecithin. On the other hand, from the more soluble fraction of the bromination product a substance was obtained which yielded unsaturated fatty acids with an iodine number of 107. This is not far removed from that required by oleic acid which has an iodine number of 90. From this fraction on hydrogenation pure stearic acid was isolated.

Assuming that only two unsaturated acids, arachidonic and oleic acids, are present in the lecithin fraction, and taking into consideration the fact that the iodine number of the mixed unsaturated acids of this lecithin is 196, it becomes easy to calculate the ratio of the two acids from the equation $90x + 335y = 196$ $(x + y)$. It follows that the ratio is approximately 1.3 parts of oleic acid to 1 part of arachidonic acid.

[2] Hartley, P., *J. Physiol.*, 1908–09, xxxviii, 353.

[3] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1921, xlviii, 185.

[4] Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 359.

The lecithin referred to above was obtained from the acetone extract of the liver. From the ethereal extract there was obtained a lecithin of lower unsaturation. The iodine number of the unsaturated acids was 136. This corresponds to 4.3 parts of oleic acid to 1 part of arachidonic acid.

Bearing in mind the molecular weight estimation of dihydrolecithin reported in the previous communication, namely 810, one is justified in concluding that the liver contains several lecithins and that the oleyl lecithins predominate.

EXPERIMENTAL.

Preparation of Material.

Beef livers in 100 lb. lots were minced, dried, and extracted, first with acetone, then with ether, and last with alcohol. These extracts were treated by the methods described in an earlier paper. A cadmium chloride salt of pure lecithin was obtained which was free from amino nitrogen.

Analysis 5. 1.0 gm. substance was hydrolyzed with HCl, neutralized, concentrated, and made up to 15 cc.

2 cc. of this solution: no N_2 (Van Slyke).

5 " " "   "   : 1.95 cc. of 0.1 N HCl (Kjeldahl).

$$\frac{\text{Amino N}}{\text{Total N}} \times 100 = 0 \text{ per cent amino nitrogen.}$$

The Unsaturated Fatty Acids.

765 gm. of the above mentioned material were hydrolyzed with 10 per cent HCl for 15 hours. The fatty acids which separated were dissolved in ether and washed with water until free from mineral acid. The ether solution was dried and concentrated to about 1.5 liters. This solution was allowed to stand at 0°C. over night. A white precipitate settled out. This was filtered off and recrystallized twice from ether and once from dry acetone. It analyzed as follows:

Analysis 15. 0.1019 gm. substance: 0.1184 gm. H_2O and 0.2834 gm. CO_2.

$C_{18}H_{36}O_2$. Calculated. C 75.93, H 12.76.

    Found.   " 75.84, " 13.00.

The ether mother liquors from which this stearic acid had been separated were then concentrated to dryness under diminished pressure. The residue was dissolved in methyl alcohol, treated with lead acetate solution, and made alkaline with ammonium hydroxide. On cooling this solution to $-5°C$. the lead salts of the fatty acids settled out. These were filtered off and extracted repeatedly with ether until ether would no longer extract material giving a precipitate with hydrochloric acid.

The combined ether extracts of the lead salts were treated with HCl to convert the salts into free fatty acids. The ether solution was then washed with water until free from mineral acid and dried with sodium sulfate. The ether was removed by distillation under diminished pressure. The residue of unsaturated fatty acids weighed 160 gm. The iodine number was obtained.

> 0.2281 gm. of substance absorbed 0.453 gm. of iodine by the Wijs method.
> Average molecular weight 290.
> Calculated (two double bonds). Iodine number 175.
> Found. " " 196.

These unsaturated acids were dissolved in glacial acetic acid and brominated with a 25 per cent solution of bromine in glacial acetic acid. The temperature was kept as low as possible without freezing the acetic acid.

The bromine solution was added from a special vacuum jacket burette constructed for the purpose. This consisted of two tubes of Pyrex glass sealed together at both ends, with a stopcock attached. The lower end of the inner tube consisted of spiral capillary tubing to take up the thermal expansion. The space between the outer and inner tubes was evacuated and sealed. Bromine solution in this burette could be kept cool longer than in an ordinary burette.

The acids were brominated in 5 to 10 gm. lots and allowed to stand over night with a slight excess of bromine. A yellow precipitate formed which was filtered off.

The precipitate (Fraction A) was expected to contain the higher bromides and the acetic acid mother liquor (Fraction B), the lower bromides.

Fraction A, the Fraction Insoluble in Glacial Acetic Acid.

Identification of Arachidonic Acid.—The precipitate was extracted repeatedly with ether until it no longer contained any ether-soluble material. This residue analyzed as follows:

Analysis 17.   0.1066 gm. substance: 0.0356 gm. H_2O and 0.0998 gm. CO_2.
           0.1996 "   " : 0.3166 gm. AgBr.
$C_{20}H_{32}O_2Br_8$.   Calculated.   C 25.43, H 3.42, Br 67.72.
         Found.   " 25.53, " 3.73, " 67.57.

In melting point determination this material darkened, melted, and decomposed at 245°C. (A slightly less pure sample of this material previously obtained contracted at 240°C. and decomposed at 244°C. while it began to darken below 200°C.)

20 gm. of the above material were obtained. 10 gm. of this were suspended in dry methyl alcohol and reduced with zinc dust and dry hydrochloric acid gas. The methyl alcohol was filtered, diluted with water, and shaken repeatedly with gasoline. The gasoline solution was then shaken with water, dried, and evaporated to dryness under diminished pressure. The iodine number of the resulting material was obtained.

0.0492 gm. substance absorbed 0.150 gm. iodine by the Wijs method.
$C_{20}H_{32}O_2$.   Calculated.   Iodine number 335.
        Found.   "   "   305.

This unsaturated material was reduced with hydrogen in alcoholic solution by the method of Paal. The solution, after being filtered, was concentrated and cooled to −5°C. The reduced acid separated out. This was recrystallized three times from dry acetone. It analyzed as follows:

Analysis 34.   0.1034 gm. substance: 0.1176 gm. H_2O and 0.2912 gm. CO_2.
$C_{20}H_{40}O_2$.   Calculated.   C 76.85, H 12.91.
        Found.   " 76.81, " 12.72.

Identification of Oleic Acid.—It was stated above that the octobromarachidic acid was extracted repeatedly with ether to remove the lower bromides. The ether mother liquors from this extraction were concentrated to 500 cc. and cooled to 0°C. A white precipitate settled out. This was separated by filtration and will be referred to as *Fraction I*.

The ethereal solution was evaporated to dryness under diminished pressure and cooled to 0°C.   Two oily layers formed.   These were separated and dissolved separately in methyl alcohol.   Each on cooling formed a sticky sediment.   These were separated. The one from the upper layer will be referred to as *Fraction II*, that from the lower as *Fraction III*.

The methyl alcohol mother liquors from Fractions II and III were then combined and reduced with zinc dust and hydrochloric acid as described above.   The resulting material weighed 30 gm. It gave an iodine value as follows:

0.2776 gm. substance absorbed 0.296 gm. iodine by the Wijs method.
$C_{18}H_{34}O_2$. Calculated.   Iodine number   90.
              Found.   "   "   107.

10 gm. of this material were reduced with hydrogen by Paal's method and recrystallized twice from dry acetone.   In order to decompose any methyl ester which might have formed during the zinc reduction the material was saponified with NaOH in methyl alcohol solution.   The soap was precipitated in acetone, dried, and converted into free acid with hydrochloric acid in the presence of ether.   The ether solution, freed from inorganic material by shaking with water, was dried and evaporated.   After three more recrystallizations from dry acetone the acid analyzed as follows:

Analysis 33.   0.0994 gm. substance: 0.1154 gm. H_2O and 0.2776 gm. CO_2.
$C_{18}H_{31}O_2$. Calculated.   C 75.93, H 12.76.
              Found.   " 76.15, " 12.99.
Melting point of stearic acid   70–71°C.
                 Found.   68°C.

Attempt to Isolate Other Unsaturated Acids than Arachidonic and Oleic Acids.—Fraction I, referred to above, was a grayish brown dry powder, difficulty soluble in ether.   For purification it was extracted with hot methyl alcohol and filtered hot.   The insoluble material analyzed as follows:

Analysis 21.   0.1062 gm. substance: 0.0374 gm. H_2O and 0.1030 gm. CO_2.
              0.2041   "   "   : 0.3030 gm. AgBr.
$C_{18}H_{30}O_2Br_4$. Calculated.   C 28.49, H 3.99, Br 63.26.
$C_{20}H_{32}O_2Br_8$.   "   " 25.43, " 3.42, " 67.72.
              Found.   " 26.46, " 4.04, " 65.26.

The alcohol extracted only a small portion of the material. When this extract was cooled to 20°C. a precipitate formed which was too small for further treatment. On cooling the mother liquor to − 5°C. a second precipitate formed. This was separated and analyzed.

Analysis 22. 0.1062 gm. substance: 0.0408 gm. H_2O and 0.1108 gm. CO_2
            0.2026 "   "   : 0.3018 gm. AgBr.
$C_{18}H_{30}O_2Br_6$. Calculated. C 28.49, H 3.99, Br 63.26.
            Found.   " 28.45, " 4.29, " 63.39.

In a melting point determination this material softened at 130°C., darkening up to 170°C. when it partially melted and partially decomposed. At 240°C. it became liquid and decomposed in the manner of octobromarachidic acid.

Since the analysis of the material approached that of a hexabromide, the substance was extracted six additional times with a large volume of methyl alcohol. In a melting point determination the residue became black at 200°C. and decomposed at 230°C. The combined extracts on cooling to 0°C. formed a precipitate which analyzed as follows:

Analysis 31. 0.2016 gm. substance: 0.3146 gm. AgBr.
$C_{18}H_{30}O_2Br_6$. Calculated. Br 63.26.
$C_{20}H_{32}O_2Br_8$.   "   " 67.72.
            Found.   " 66.41.

In a melting point determination this material softened at 140°C., darkening with rise of temperature, and melting with decomposition at 243°C.

The methyl alcohol mother liquors were concentrated to 100 cc. and cooled to 0°C. A second precipitate formed which melted as follows. It softened at 140°C., partially decomposed at 180°C., and melted with decomposition at 243°C.

Thus each fraction obtained from Fraction I on purification approached the character of the octobromide, hence it is justifiable to assume that this fraction consisted mainly of the octobromide.

It was next attempted to isolate a tetrabromide from the material referred to as Fractions II and III (page 290). Each was dissolved in excess of ether and treated with 5 parts of gasoline.

Precipitates were formed in each and were separated. These had a sticky consistency which indicated that they were not pure.

They were obtained in a quantity too small for analysis. The combined gasoline mother liquors were concentrated to small volume and again treated with gasoline. A precipitate was formed which analyzed as follows:

Analysis 25.   0.1003 gm. substance: 0.0408 gm. H_2O and 0.1118 gm. CO_2.
                     0.1972 "   "   : 0.2806 gm. AgBr.
$C_{18}H_{32}O_2Br_4$.   Calculated.   C 36.01, H 5.38, Br 53.28.
$C_{18}H_{30}O_2Br_6$.   "   " 28.49, " 3.99, " 63.26.
                     Found.   " 30.39, " 4.55, " 60.56.

The mother liquors from this precipitate were again concentrated, dissolved in dry methyl alcohol, and reduced with zinc dust and hydrochloric acid. The reduced material had an iodine number as follows:

0.1990 gm. substance absorbed 0.264 gm. iodine by the Wijs method. Found. Iodine number 123.

The reduced material was dissolved in glacial acetic acid and rebrominated. This bromine will be referred to as *Fraction IV*.

The acetic acid solution was concentrated to dryness under diminished pressure. The residue was dissolved in hot absolute alcohol and cooled to −5°C. A precipitate formed which was again dissolved in hot absolute alcohol and reprecipitated by cooling to −5°C. After reprecipitating eight times from absolute alcohol and once from methyl alcohol, a melting point determination was made. It softened at 150°C. and melted with decomposition at 240°C. Apparently this is an impure octobromide.

The mother liquors were concentrated and cooled to − 5°C. A precipitate was formed which, after several reprecipitations from absolute alcohol and from methyl alcohol, melted as follows: At 130°C. it softened and became black. At 230°C. it melted with decomposition. This also appears to be an impure octobromide.

In order to ascertain whether a tetrabromide was present in the mother liquors from the purification of the last substance they were concentrated to dryness under diminished pressure and dissolved in gasoline. Since no precipitate formed even on cooling to −5°C., it was concluded that no tetrabromide was present.

The three precipitates obtained from Fractions II and III were combined and also reduced with zinc dust and hydrochloric acid. The reduced material gave an iodine number as follows:

0.2815 gm. substance absorbed 0.488 gm. iodine by the Wijs method. Found. Iodine number 180.

This material was also rebrominated in glacial acetic acid solution. It will be referred to as *Fraction V*. The bromide solution was treated in the same way as Fraction IV. It yielded similar results. Purification of the insoluble material gave an octobromide.

Fraction B, the Fraction Soluble in Acetic Acid.

This consists of the acetic acid mother liquors from which Fraction A was filtered. The liquors were concentrated under diminished pressure and treated with gasoline. An oily layer settled out. This was separated, dissolved in methyl alcohol, and reduced with zinc and hydrochloric acid. 20 gm. of reduced material were formed having an iodine number as follows:

0.1974 gm. substance absorbed 0.356 gm. iodine by the Wijs method. Found. Iodine number 180.

The acids were rebrominated in glacial acetic acid. These bromides (*Fraction VI*) were treated in the same manner as Fractions IV and V and yielded similar material. Had there been any tetrabromides they would have been found in Fractions IV, V, and VI.

Lecithin Containing a Smaller Percentage of Highly Unsaturated Acids.

A sample of lecithin was obtained from the alcohol-insoluble portion of the ether extracts of liver. This was purified by dissolving in acetic acid and treating with alcohol. The solution after being separated from the precipitate which formed was concentrated under diminished pressure, emulsified with water, and precipitated with acetone. This precipitate was dissolved in alcohol and cooled to 0°C. A precipitate formed. The solution was separated, concentrated under diminished pressure, dissolved in ether, and precipitated with acetone.

This precipitate was converted into the cadmium chloride salt and purified once by the ether crystallization method. The purified compound was converted into free lecithin by means of ammonia in methyl alcohol solution. The lecithin was again converted into the cadmium chloride salt and again purified by the ether crystallization process, using a large volume of ether. This material was free from amino nitrogen.

About 1.0 gm. of material was dissolved in 15 cc. of glacial acetic acid. 2 cc. of this solution: no N (Van Slyke).
5 "   "   "   "   : 1.35 cc. 0.1 N HCl (Kjeldahl).

This cadmium chloride salt of lecithin was then hydrolyzed with 10 per cent HCl. The iodine number was determined on the fatty acids after they had been freed from mineral matter and dried.

0.2120 gm. substance absorbed 0.256 gm. iodine by the Wijs method. Found. Iodine number 65.

These acids were then converted into the lead salts and extracted with ether. The ether-soluble lead salts were converted into free acids with HCl. Their iodine number was determined.

0.2672 gm. substance absorbed 0.363 gm. iodine by the Wijs method. Found. Iodine number 136.

CARBONIC ACID AND BICARBONATE IN URINE.

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INTRODUCTION.

In the course of some studies of acid excretion undertaken in this laboratory it became desirable to learn something if possible, of the factors which determine the elimination of carbonic acid in urine. Although carbonic acid may be regarded as only incidentally a urinary acid, the bulk of its production leaving the body by way of the lungs, it is, nevertheless, regularly present in urine, both free and bound as bicarbonate. Appraisement of the extent to which it may enter the urine is, therefore, necessary from the point of view of an understanding of the factors controlling the reaction of the urine, and is also required in quantitative studies of the acid-base content of urine.

To explain the presence of H_2CO_3 in urine several conjectures are permissible: (*a*) that H_2CO_3 enters the urine from the blood plasma at a regulated rate, *i.e.* is secreted by the kidney; (*b*) that $BHCO_3$[1] entering the urine reacts with acid phosphate and liberates H_2CO_3 to such an extent as will be determined by the phosphate concentration; and (*c*) that H_2CO_3 diffuses readily through renal epithelium with the result that the concentration of H_2CO_3 in the urine is determined by that obtaining in the blood plasma. Considering the inappreciable part of the total H_2CO_3 production conveyed from the body in the urine, its volatile character, and the fact that its concentration in the plasma is regulated by the respiratory mechanism, an active control of its elimination by the kidney seems unlikely. That the renal epithelium is no barrier

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[1] B represents a univalent atom of base. This convenience in notation is taken from Van Slyke (1).

to its obedience to the gas laws is a much easier assumption.   An equilibrium between the tension of CO_2 in urine and in plasma does not, of course, preclude the derivation of H_2CO_3 from bicarbonate in the urine (see (*b*) above).

The data presented in this paper were obtained with the purpose of testing the hypothesis of a direct relationship between H_2CO_3 concentration in urine to that obtaining in blood plasma.   If the CO_2 tension of urine is determined by the CO_2 tension of plasma, then both the H_2CO_3 and $BHCO_3$ concentrations in urine are definitely indicated. H_2CO_3 will remain a nearly constant value, the variation of its concentration in urine being no greater than in plasma, regardless of variation of the reaction of urine.   The concentration of H_2CO_3 remaining stationary, or nearly so, the $BHCO_3$ concentration must then vary exactly inversely with the urinary pH.[2]   In other words $BHCO_3$ concentration will become a linear function of pH.   Urine will thus always contain at a given pH approximately the same amount of bicarbonate.   The size of the $BHCO_3$ concentration at a given pH will be in such proportion to the value for the stationary H_2CO_3 concentration as is determined by the $\dfrac{H_2CO_3}{BHCO_3}$ ratio which must obtain at this pH.   The range of variation of $BHCO_3$ concentration at a given pH should be relatively no greater than the slight variation (under normal circumstances of metabolism) of the H_2CO_3 concentration in plasma. The total amount of carbonic acid (H_2CO_3 + $BHCO_3$) entering the urine will, owing to the variation of $BHCO_3$ with pH, be determined by the reaction of the urine, with the result that the total carbonic acid elimination in urine will decrease with increase in urinary acidity.   If the above outlined consequences of a direct relationship between the CO_2 tension of the plasma and the CO_2 tension of the urine actually obtain they should be easily demonstrable by measuring in a series of urine specimens, the concentrations of H_2CO_3, $BHCO_3$, and pH.   The finding regularly in urine of the values for H_2CO_3 and $BHCO_3$ postulated above would

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[2] The pH of solutions containing carbonic acid and bicarbonate being determined by the ratio of the concentrations of these substances, it follows that if one of the three factors in this equilibrium, in this instance (H_2CO_3), be immobilized, the other two, ($BHCO_3$) and pH, must necessarily vary exactly inversely.

constitute satisfactory evidence for regarding carbonic acid elimination in urine as a consequence of the CO_2 tension in blood plasma. Such measurements obtained from a series of 55 urine specimens are presented below.

Methods.

Collection of Urine.—It was found to be a matter of essential importance that the urine be collected in such a way as to avoid as nearly as possible loss of CO_2. The free carbonic acid tends to leave the urine rapidly after voiding. In consequence of the loss of H_2CO_3 there occurs a diminution also of $BHCO_3$ with shift of base to acid phosphate. Measurement of the H_2CO_3 and $BHCO_3$ content of 24 hour urine collections necessarily gives low and irregular values.[3] It was decided to use in this study only freshly voided specimens. In order to minimize loss of CO_2 the voidings were collected in large test-tubes fitted with long stemmed funnels which delivered the urine in the bottom part of the tube. The tubes were nearly filled with urine and then immediately stoppered.

Measurement of pH.—The pH of the specimen was determined colorimetrically reading to the nearest first decimal numeral of the logarithmic notation scale. Undiluted urine was used and comparison with the standard· carried out by the "comparator method," of Walpole, *i.e.* a tube containing urine was placed behind the standard and a tube containing distilled water behind the urine sample receiving the indicator. Phosphate standards were used, the accuracy of which had been tested by potentiometer readings.

Measurement of Total CO_2.—Total CO_2 was determined in the Van Slyke apparatus for measuring the CO_2 content of plasma. The sample was taken from the bottom portion of the contents of the collecting tube and was introduced under 1 per cent NH_4OH solution into the cup at the top of the burette. The sample was measured between marks on the pipette so that the upper part of the urine column, from which occurs the greatest loss of CO_2, was left in the pipette. In order to obtain a satisfactory burette reading it was necessary to use samples of various sizes; *viz.*, 5, 2, 1, and 0.25 cc. It was found that the amount to be taken was indicated by the pH of the urine. A single extraction was made and the burette reading

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[3] The only extensive study of the CO_2 content of urine which has been reported is that of Denis and Minot (2). Their specimens were 24 hour collections which were kept in well stoppered containers. There was, however, apparently no precaution taken to prevent an initial loss of CO_2. Estimation of H_2CO_3 and $BHCO_3$ from their data, which include also a measurement of pH, gives results which are widely variable as compared with the values presented in this paper. It is believed that the discrepancy must be due to loss of CO_2 from their specimens during voiding and while the urine was being transferred to the container.

corrected for CO_2 remaining in samples after a single extraction and for the air content of the samples. The factors for correction for unextracted CO_2 were calculated from the ratio of the burette capacity (50 cc.) to the volume of the samples plus the 1.5 cc. of reagents. The factors used for the amounts given above were 1.15, 1.075, 1.05, and 1.02, respectively. Their accuracy was tested by a series of double extractions. The value for air content of urine plus reagents was directly determined in a series of specimens by absorbing the CO_2 after extraction with 5 per cent KOH. The total air content, depending on the size of sample used, was found to be 0.11, 0.06, 0.04, or 0.02 cc. After multiplying the burette reading by the factor for unextracted CO_2, the appropriate value for total air content was subtracted and a third correction for temperature $\left(\dfrac{273}{273 + T} \right)$ then applied.

Calculation of "Free" and "Bound" CO_2.—Fig. 1 represents graphically the $\left(\dfrac{H_2CO_3}{BHCO_3} \right)$ ratio over the range of urinary pH. The curve was obtained from the equation pH $= 6.1 + \log \dfrac{BHCO_3}{H_2CO_3}$.[4] In Table I is given the percentage of the total CO_2 which is free at various degrees of pH. These values were read from the curve (Fig. 1) and were used in estimating the H_2CO_3 and $BHCO_3$ content of the urine specimens after measurement of pH and total CO_2 content.

Accuracy of Measurements.—The accuracy of the measurements of free and bound CO_2 in urine depends in the first instance on the prevention of loss of CO_2 from the specimen while it is being collected. The collection error with the technique used cannot, unfortunately, be directly determined. The necessity for the precautions taken and the size of the possible error in estimating H_2CO_3 without them is indicated by the data in Table II, in which the results obtained by analysis of urine voided into a beaker are compared with those obtained from a portion of the same voiding collected in the manner described. The analysis of the urine voided into the beaker was carried out immediately, *i. e.* the sample for analysis was introduced into the burette within a few moments after voiding the specimen. The large error in the H_2CO_3 measurement due to loss of CO_2 is apparent. In the case of the specimens in which the phosphate concentration is low, this error is due not only to the direct loss of CO_2 but to a consequent alkaline shift of reaction. Decrease in pH greatly reduces the calculated value for H_2CO_3 (see Table I).

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[4] Hasselbalch's equation. For derivation of this equation see Van Slyke (1).

Owing to the wide variation of $BHCO_3$ concentration in urine the extent to which it dissociates does not remain a constant. This equation with its fixed value for pK_1 will, therefore, measure the H_2CO_3: $BHCO_3$ ratio with only an approximate accuracy.

It should be admitted that the technique used probably did not prevent a very slight loss of CO_2. The values obtained in the most acid urine specimens in which the total CO_2 is a very small value are possibly somewhat low for this reason.
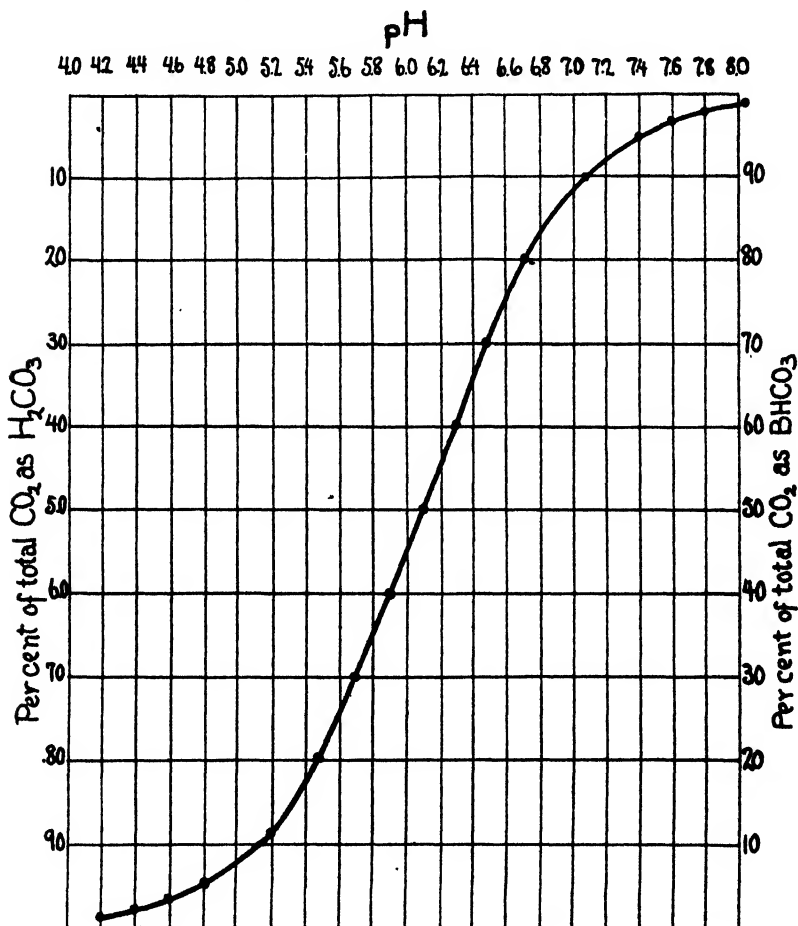
FIG. 1. Curve indicating per cent of total CO_2 which is free or bound at various degrees of hydrogen ion concentration.

A much more serious obstacle to an accurate estimation of H_2CO_3 and $BHCO_3$ is encountered in the wide range of the total CO_2 content of urine. This range may be indicated as approximately 1 to 100 between pH 5.0 and pH 8.0. pH readings to the first decimal place give 30 points over

this range. The pH reading on which depends the partition of the total CO_2 into free and bound CO_2 is, therefore, not closely enough determined to provide more than an approximate accuracy in the estimation of H_2CO_3 and $BHCO_3$. This difficulty is increased over the middle part of the pH

TABLE I.

Percentage of Total CO_2 as H_2CO at Various Degrees of Hydrogen Ion Concentration.

pH	Free CO_2 as per cent of total CO_2.	pH	Free CO_2 as per cent of total CO_2.	pH	Free CO_2 as per cent of total CO_2.
5.0	93	6.0	55	7.0	12.0
5.1	91	6.1	50	7.1	9.5
5.2	89	6.2	45	7.2	8.0
5.3	86	6.3	40	7.3	6.5
5.4	83	6.4	35	7.4	5.0
5.5	80	6.5	30	7.5	4.0
5.6	75	6.6	25	7.6	3.0
5.7	70	6.7	20	7.7	2.5
5.8	65	6.8	17	7.8	2.0
5.9	60	6.9	14	7.9	1.5

TABLE II.

Showing Error in Estimation of H_2CO_3 Due to Loss of CO_2 from the Urine Specimen.

Specimen.	Method of collection.	pH	Burette reading.	H_2CO_3	HPO_4
			cc.	vol. per cent CO_2	gm. mol.
1	Test-tube,	5.2	0.32	4.3	0.028
	Beaker.	5.2	0.28	3.5	
2	Test-tube.	6.3	0.70	5.2	0.018
	Beaker.	6.3	0.53	3.7	
3	Test-tube.	6.6	0.98	4.7	0.002
	Beaker.	6.8	0.90	2.9	
4	Test-tube.	7.1	0.60	5.2	0.008
	Beaker.	7.3	0.56	3.3	

range by the rapidly shifting character of the $\left(\dfrac{H_2CO_3}{BHCO_3}\right)$ ratio. Inspection of the curve, Fig. 1, and the values in Table I will make these points clear.

*Evidence Indicating Direct Relationship of the Elimination of
Carbonic Acid in Urine to the H_2CO_3 Concentration
in Plasma.*

As indicated above, measurements of the H_2CO_3 and $BHCO_3$ content of urine carried out in the manner described are to be regarded as of only an approximate accuracy. Collected, however, from a fairly large series of urine specimens over a wide range of pH· they serve satisfactorily the purpose of this study which is a statistical rather than direct attempt to learn whether or not there is a direct relationship of CO_2 elimination in the urine to the CO_2 tension of plasma. In estimating the significance from this point of view of the values for H_2CO_3 and $BHCO_3$ found in urine, it should be remembered that since the H_2CO_3 concentration in plasma varies normally to the extent of about $\pm$ 15 per cent of its average value, we may expect the same degree of variation in the values for H_2CO_3 and $BHCO_3$ in urine prescribed by the assumption that they are determined by the CO_2 tension of plasma.

The values for total, free, and bound carbonic acid found in urine over a range of pH extending from 5.0 to 8.0 are given in Table III. Inspection of these measurements shows very clearly that the H_2CO_3 concentration in all of these specimens is a fairly constant value and bears no relation to pH. The average of the measurements of H_2CO_3 concentration is 4.2 volumes per cent CO_2. Variation from this average is $\pm$ 25 per cent. In contrast with this fairly steady value for H_2CO_3, $BHCO_3$ concentration varies over an enormous range and, moreover, is directly related to pH, the thousandfold increase in pH from 8.0 to 5.0 being accompanied by an approximately thousandfold diminution of $BHCO_3$. In consequence of this decrease in bicarbonate with increase in acidity of the urine there is also a rapid fall in the total amount of carbonic acid (free + bound) entering the urine. This large decrease in the total CO_2 content of urine which accompanies increase in pH has been shown by Denis and Minot (2). In order to demonstrate that the regularity of the relationship of the total CO_2 content of urine to pH is independent of variation in the phosphate concentration the data in Table IV are presented.

These findings will excellently suit the hypothesis that carbonic acid elimination in urine is simply a consequence of the CO_2 tension of plasma provided it can be shown that the nearly

TABLE III.

Measurements of the Total Bound and Free Carbonic Acid Content, and pH of 55 Urine Specimens.

Subject.	pH	Total.	$BHCO_3$	H_2CO_3	Subject.	pH	Total.	$BHCO_3$	H_2CO_3
		vol. per cent CO_2	*vol. per cent CO_2*	*vol. per cent CO_2*			*vol. per cent CO_2*	*vol. per cent CO_2*	*vol. per cent CO_2*
R.	8.0	430*	426	4.3	R.	6.6	16.6	12.4	4.2
					G.	6.6	16.2	12.1	4.1
R.	7.9	350*	345	5.3	G.	6.6	16.0	12.0	4.0
					G.	6.6	15.9	11.9	4.0
G.	7.8	222*	218	4.4					
						6.5			
	7.7				H.	6.4	12.9	8.4	4.5
G.	7.6	174*	169	5.2	G.	6.4	12.9	8.4	4.5
	7.5				G.	6.3	12.1	7.3	4.8
					G.	6.3	11.8	7.0	4.8
G.	7.4	94*	89	4.7	G.	6.3	11.7	7.0	4.7
G.	7.4	88	74	4.4					
G.	7.3	71.8*	67.1	4.7	W.	6.2	10.8	5.9	4.9
G.	7.3	64.8	60.6	4.2	S.	6.2	9.7	5.3	4.4
G.	7.3	60.2*	56.3	3.9	H.	6.1	9.5	4.7	4.8
G.	7.2	56.5	52.0	4.5	C.	6.0	8.5	3.8	4.7
G.	7.2	51.4	47.3	4.1					
T.	7.1	49.0	44.3	4.7	R.	5.9	7.3	2.9	4.4
G.	7.1	48.8	44.2	4.6	G.	5.9	6.5	2.6	3.9
G.	7.1	45.2	40.9	4.3					
G.	7.1	43.0	38.9	4.1	G.	5.8	6.3	2.2	4.1
G.	7.0	39.4	34.7	4.7	G.	5.7	5.9	1.8	4.1
G.	7.0	38.3†	33.7	4.6	G.	5.7	5.3	1.6	3.7
G.	7.0	35.5	31.2	4.3	G.	5.6	5.6	1.4	4.2
G.	7.0	35.3	31.1	4.2	G.	5.6	5.4	1.4	4.0
G.	6.9	36.7†	31.6	5.1	K.	5.4	4.4	0.7	3.7
G.	6.9	30.1	25.9	4.2					
G.	6.9	27.3	23.5	3.8	T.	5.3	4.4	0.6	3.8
					B.	5.3	4.0	0.6	3.4
G.	6.8	25.3†	21.0	4.3	G.	5.3	4.0	0.6	3.4
G.	6.8	21.7	18.0	3.7					

* After ingestion of $NaHCO_3$.

† After ingestion of Na_2HPO_4.

Subject.	pH	Total.	BHCO₃	H₂CO₃	Sub-ject.	pH	Total.	BHCO₃	H₂CO₃
		vol. per cent CO_2	vol. per cent CO_2	vol. per cent CO_2			vol. per cent CO_2	vol. per cent CO_2	vol. per cent CO_2
					F.	5.2	4.4	0.5	3.9
T.	6.7	19.8	15.8	4.0	W.	5.2	3.7	0.4	3.3
G.	6.7	19.8	15.8	4.0					
					R.	5.0	3.7	0.2	3.5
					D.	5.0	3.5	0.2	3.3
					K.	5.0	3.5	0.2	3.3

constant H_2CO_3 concentration in urine is of an appropriate size in relation to the same value in plasma. The basis of the CO_2 tension of the body fluids is the CO_2 tension maintained in the alveolar air by the respiratory mechanism. The average CO_2 tension in alveolar air is given as 45 mm. Dividing 45 by 760 this value becomes 5.9 volumes per cent CO_2. Multiplying 5.9 by 0.54 (the absorption coefficient of CO_2 in blood serum given by

TABLE IV.

Molecular Concentration of—HCO_3 *and*—HPO_4 *in Urine in Relation to pH.*

Subject.	pH	— HCO₃	— HPO₄	Subject.	pH	— HCO₃	— HPO₄
		M	M			M	M
R.	7.9	0.157	0.016	T.	6.9	0.009	0.017
G.	7.6	0.078	0.006	C.	6.0	0.004	0.028
T.	7.1	0.022	0.018	G.	5.3	0.002	0.017
G.	6.9	0.016	0.040	B.	5.3	0.002	0.040
G.	6.7	0.009	0.002	T.	5.3	0.002	0.045

Bohr[5]), 3.2 volumes per cent CO_2 are indicated as the average H_2CO_3 concentration in blood plasma. The higher concentration (4.2 volumes per cent CO_2) found in urine may be explained by accepting it as evidence of a higher CO_2 tension in renal tissue than obtains in plasma. Urine during its passage through the tubules of the kidney would quite certainly acquire the CO_2 tension of renal tissue. A few direct measurements of the CO_2 tension of urine have been recorded. Fredericq (3), using a micro

[5] Cited from Van Slyke (1).

tonometer found that the CO_2 tension in urine, collected without exposure to air, from dogs was in average (6 observations) 11 per cent of an atmosphere.   Strassburg (4), a much earlier worker, gives as the result of three measurements 9 per cent of an atmosphere as the CO_2 tension in the urine of dogs.   Assuming that the absorption coefficient of CO_2 in urine does not appreciably differ from that in plasma[6] these values indicate 5.9 and 4.9 volumes per cent CO_2 as H_2CO_3.   They are thus still larger in relation to the usual concentration of H_2CO_3 in plasma than the average value found in this study, and may be taken to indicate that the CO_2 tension in renal tissue is higher than in plasma.   There is thus the probability that the H_2CO_3 concentration of urine as it enters the bladder is determined by a CO_2 tension which is somewhat higher than obtains in plasma.   Presumably, however, this higher tension to which the urine is exposed in the kidney is directly proportional to the CO_2 tension of plasma and we may, therefore, correctly regard the elimination of CO_2 in the urine as a diffusion process controlled by the tension of the gas in the plasma.

Relation of Carbonic Acid to the Reaction of Urine.

Accepting 4.2 volumes per cent CO_2 as an approximate estimation of the average concentration of H_2CO_3 in urine, the average $BHCO_3$ content of urine at a given pH may be readily calculated by reference to the $\dfrac{H_2CO_3}{BHCO_3}$ ratio obtaining at this pH.   The estimated average bicarbonate content of urine at frequent points in the range of urinary pH, expressed as volume per cent CO_2 and also as cc. of 0.1 N base and as gm. of $NaHCO_3$ per liter, is given in Table V.   It will be noted that the values expressed as volume per cent CO_2 agree approximately with the direct measurements of $BHCO_3$ (Table III).   It is desired to call attention by means of this table to the increasingly huge amounts of bicarbonate which are required to force the reaction of the urine in the direction of alkalinity.   Fig. 2 is presented as further illustration of this point. Evidently the maintenance in the urine of a constant H_2CO_3 con-

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[6] The solubility coefficient in water at body temperature is given by Bohr as 0.555 (cited from Van Slyke, 1). CO_2 is thus only slightly less soluble in blood serum than in water.   In urine it is doubtless not less soluble than in serum nor more soluble than in water.

centration offers resistance to an alkaline shift of reaction which becomes more and more effective as the amount of base entering the urine increases. For example at pH 7.4 the urine contains 3 gm. of bicarbonate per liter. In the absence of H_2CO_3 this large concentration of bicarbonate would force down the pH of the urine to 7.9 or 8.0. The small amount of acid phosphate in urine at pH 7.4 could only slightly check the shift. With the relatively small H_2CO_3 concentration present in urine about 15 gm. of bicarbonate per liter are required to force the reaction to pH 8.0.

It should be noted that the maintenance in the urine of a constant H_2CO_3 concentration will offer effective resistance to depression of pH due to base carried into the urine as alkaline phosphate. The events which will transpire following the arrival in urine of alkaline phosphate, H_2CO_3 being maintained, may be breifly stated as follows: H_2CO_3 will deprive B_2HPO_4 of some of its base to form $BHCO_3$ thus releasing a certain amount of BH_2PO_4. This proc-

TABLE V.

Estimated Bicarbonate Content of Urine, Assuming 4.2 volumes per cent CO_2 Unbound.

pH	BHCO$_3$			pH	BHCO$_3$		
	vol. per cent CO_2	cc. 0.1 N	gm. $NaHCO_3$ per liter		vol. per cent CO_2	cc. 0.1 N	gm. $NaHCO_3$ per liter
7.8	206	920	7.70	6.4	7.8	35	0.29
7.6	136	600	5.10	6.2	5.1	23	0.19
7.4	80	360	3.00	6.0	3.4	15	0.13
7.2	48	216	1.80	5.8	2.3	10	0.08
7.0	31	140	1.15	5.6	1.4	6	0.05
6.8	20.5	92	0.77	5.4	0.9	4	0.03
6.6	12.6	56	0.47	5.2	0.5	2	0.02

ess will go on until the $\dfrac{BH_2PO_4}{B_2HPO_4}$ and $\dfrac{H_2CO_3}{BHCO_3}$ ratios stand in equilibrium, and the basis of this equilibrium, it should be noted, will be the "normal" H_2CO_3 concentration.

Finally it should be pointed out that the reaction of the urine of a pH below 6.8 or 7.0 becomes largely a function of the $\dfrac{H_2CO_3}{BHCO_3}$ ratio rather than of the $\dfrac{BH_2PO_4}{B_2HPO_4}$ ratio which as Henderson has

pointed out determines the reaction of urine over the usual range of pH in urine. This being the case, a large alkaline shift of reaction is likely to occur after voiding, in specimens of a pH
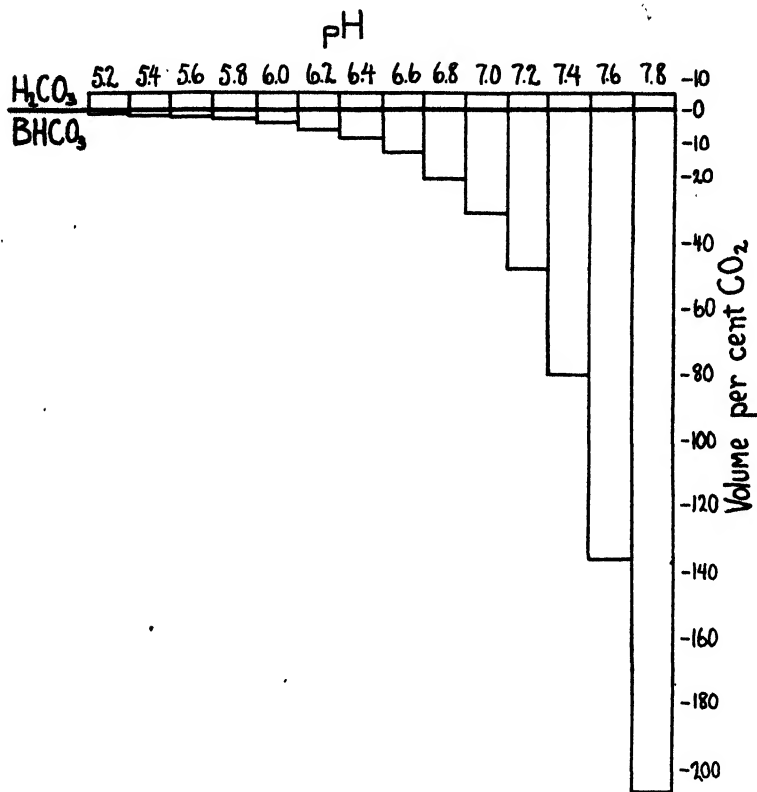
FIG. 2. Diagram of the H_2CO_3 and $BHCO_3$ concentrations in urine at various degrees of hydrogen ion concentration constructed on the basis of a constant H_2CO_3 concentration of 4.2 volumes per cent CO_2.

between 7.0 and 8.0, unless the specimens are collected with precautions against loss of CO_2. This point has already been indicated in Table II, and is of practical importance in any study of acid excretion depending on a measurement of pH.

Significance of the Usual Acidity of Urine from the Standpoint of Economy in Base Elimination.

In the process of conveyance into the urine of the acid end-products of metabolism it is necessary that base be used with a regulated economy. Henderson (5) has pointed out the large economy of base in the elimination of phosphoric acid due to the fact that the pH of urine is usually much greater than that of blood plasma. The manner of this saving of base can be briefly described. Phosphoric acid is bound both in the body fluids and in urine as acid phosphate and as di-basic phosphate. In the body fluids at pH 7.4 the ratio of $BH_2PO_4 : B_2HPO_4$ is 1:4, whereas in urine of average reaction, pH 6.0, the ratio is more than reversed, becoming 9:1. From these ratios it may be calculated that the base bound by phosphoric acid while being conveyed for excretion is 1.8 times (in terms of normality) the molecular concentration of HPO_4, whereas in urine at pH 6.0 the base equivalent is 1.1. The elimination of phosphoric acid in urine of pH 6.0 is thus accomplished with a saving of approximately 40 per cent of the base bound by HPO_4 in the body fluids. There is also a slight economy of base in the elimination of the organic acids normally present in urine due to the fact that a small part of their total concentration is unbound at the usual reaction of urine. In the presence of ketosis, with a large elimination of organic acids, this base economy although remaining small in relation to the total organic acid excretion, may equal in size the economy obtained in the excretion of phosphoric acid. A glance at Table V will at once reveal the fact that by the elimination of urine at pH 6.0 instead of at pH 7.4, there is effected a large, and indeed a nearly complete, saving of base as bicarbonate.

In Table VI is given a quantitative comparison of the extent of base economy in the elimination of phosphoric acid, the organic acids, and carbonic acid. These data were provided by a young girl (an epileptic) during the 5th day of a fast, undertaken as a therapeutic measure. Ketosis due to starvation was on this day at its height. The figures given were obtained from a 24 hour urine specimen. Base economy was measured by subtracting the base bound by these several acid substances at the pH of the urine specimen, 5.3, from the amount of base which they would bind at the reaction of the blood plasma, pH 7.4. In the case of phosphoric acid the factors for this calculation were obtained from the

phosphate ratios at pH 5.3 and pH 7.4 using Sörensen's data. The free organic acid value representing the economy in the elimination of these substances was obtained by subtracting from the titratable acidity of the urine (to pH 7.4) the part of this measurement which is due to acid phosphate; *viz.*, the 239 cc. already indicated in the table as the measure of base economy in the elimination of phosphoric acid.[7] The bicarbonate values were estimated from the volume of the specimen and the approximate data given in Table V. The table is completed as a balance sheet of acid-base excretion by adding an estimation of total acid excretion, including the acid radicles SO_4 and Cl which carry their full equivalents of base into the urine, in order that the size of the total base economy and ammonia production may be compared with total acid excretion and the extent to which these two factors have limited inorganic base excretion measured.

The chief purpose in presenting Table VI is to indicate by actual measurement that, with an acid urine, the economy of base as bicarbonate is much greater than the economy of base effected in the elimination of phosphoric acid or of the organic acids. In this instance it is found to be twice as large as the combined saving of these two other factors. It may also be noted that this saving alone is nearly as large as the total amount of inorganic base which was permitted to enter the urine. This much larger economy is due not only to the decrease in acid urine of the proportion of the total carbonic acid which is bound but also to the fact that in acid urine the total carbonic acid content is a small value. These two causes practically eliminate bicarbonate from very acid urines. This point will be made quite clear by reference to Table III. The saving of base spent as bicarbonate is indeed nearly complete at the usual reaction of the urine. The negligible amount of bicarbonate in urine in the neighborhood of pH 6.0 compared with the large bicarbonate content at pH 7.4 is apparent in Table V and Fig. 2. Inasmuch as carbonic acid can be carried out of the body base-free by way of the lungs, base entering the urine as bicarbonate must, from the standpoint of the economics of acid-base elimination, be regarded as base totally wasted. By the elimination of urine at its usual degree of acidity

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[7] The organic acid elimination was measured by the method of Van Slyke and Palmer (6) and the titratable acidity by the method of Palmer and Henderson (7).

a large and entirely useless expenditure of base as bicarbonate is almost completely avoided. This fact is a chief significance of the usual reaction of urine.

<div align="center">TABLE VI.</div>

Base Economy in Elimination in Urine of Phosphoric Acid, Organic Acids, and Carbonic Acid.

Data from 16 year old girl during 5th day of a fast.

Measurements in urine (24 hour collection).

Volume 2,300 cc. pH 5.3. Titratable acidity (to pH 7.4) 473 cc. 0.1 N.

Organic acids, 1,641 cc. 0.1 N.

Phosphates (inorganic), 0.951 gm. P = 306 cc. .0.1 M HPO_4.

Sulfates (inorganic), 0.461 gm. S = 290 cc. 0.1 N SO_4.

Chlorides, 1.02 gm. Cl = 292 cc. 0.1 N Cl.

Ammonia, 1,370 cc. 0.1 N.

Estimations of base economy.

Base bound in urine by:

$-HPO_4$ at pH 7.4, 306 × 1.8 = 551 cc. 0.1 N.

" " " 5.3, 306 × 1.02 = 312 " 0.1 "

Base economy = 239 " 0.1 "

Organic acids at pH 7.4 = 1,641 cc. 0.1 N.

" " " " 5.3 = 1,407 " 0.1 "

Base economy = 234 " 0.1 "

$-HCO_3$ at pH 7.4, 2.3 × 360 = 828 cc. 0.1 N.

" " " 5.3, 2.3 × 3 = 7 " 0.1 "

Base economy = 821 " 0.1 "

Total acid excretion (in terms of base bound).

	pH 7.4	*pH 5 3*
Organic acid	1,641 cc. 0.1 N.	1,407 cc. 0.1 N
$-HCO_3$	828 " 0.1 "	7 " 0.1 "
$-HPO_4$	551 " 0.1 "	312 " 0.1 "
$-SO_4$	290 " 0.1 "	290 " 0.1 "
$-Cl$	292 " 0.1 "	292 " 0.1 "
Total	3,602 " 0.1 "	2,308 " 0.1 "

Total base at pH 7.4................................3,602 cc. 0.1 N

" " " " 5.3.................................2,308 " 0.1 "

" " economy.....................................1,294 " 0.1 "

" " at pH 5.3..................................2,308 " 0.1 "

Ammonia production.......................................1,370 " 0.1 "

Total inorganic base in urine........................ 938 " 0.1 "

CONCLUSIONS.

Measurements of pH and of the concentrations of free and bound carbonic acid in a series of 55 urine specimens collected in such a manner as to prevent as nearly as possible loss of CO_2 permit the following statements. Free carbonic acid in urine is a nearly stationary value. The bicarbonate content of urine varies inversely with the urinary pH. In consequence of the nearly stationary value for the numerator of the $\dfrac{H_2CO_3}{BHCO_3}$ ratio the bicarbonate content of urine at a given pH is an approximately constant value. The total carbonic acid content of urine (free + bound) falls rapidly with increase in pH, owing to the diminution of bicarbonate which accompanies rise in pH. These findings permit the inference that the elimination of carbonic acid in urine is determined by the CO_2 tension of blood plasma.

Maintenance in the urine of an approximately constant concentration of free carbonic acid greatly limits alkaline shift in the reaction of urine following increase in base elimination. The reaction of urine of a pH below 7.0 is much more a function of the carbonic acid-bicarbonate ratio than of the ratio of the phosphates. The reaction of voided urine may, therefore, rapidly increase in alkalinity because of loss of free carbonic acid unless collected with precaution against this loss.

Owing to the rapid diminution of the bicarbonate content of urine with rise in pH, urine of the usual degree of acidity contains a very small amount of bicarbonate. This fact represents avoidance of a large and altogether wasteful expenditure of base.

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STUDIES OF THE METABOLISM OF DIABETES.*

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CONTENTS.

INTRODUCTION.

A patient with diabetes mellitus, under observation in the Mayo
Clinic from October 4, 1920 to October 3, 1921, has undergone
two courses of fairly complete metabolic study. The latter of
these continued without interruption for 11 weeks. At no time
during the year was this patient observed to utilize more than 30
gm. of glucose derivable from the carbohydrate and protein of
the food and twice during the longer period of observation, when
the diet consisted almost exclusively of protein and fat, the D:N
ratio of the urine rose to 3.65, indicating complete diabetes.

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Connecticut, December 30, 1921.

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The extent and character of the investigations of this case, and the length of the period of study, warrant this detailed report particularly since the number of controlled observations of patients exhibiting a 3.65 ratio is small and since the significance of the ratio is still a matter of dispute. The studies bear, furthermore, on the effect of high protein and high fat dietaries and on other phases of diabetic metabolism particularly the basal metabolic rate and the respiratory quotient.

Methods of Study, Analyses, and Calculations.

We endeavored to approach the same accuracy in the matter of controlling the food intake and the collection of excreta as is maintained in the metabolism ward of the Russell Sage Institute, described by Gephart and Du Bois (18). The patient was cared for in a private room adjacent to the laboratory and during the crucial periods of observation she was not allowed to leave the room. The composition of the diets was calculated from Atwater and Bryant's (4) tables, the individual constituents being weighed with an error of less than 1 gm. The foods were as simple as the length of the periods of observation permitted, and consisted of string beans, asparagus, tomatoes, eggs, cream of known composition, butter, tenderloin steak, and a soy bean bread. At no time was food left uneaten by the patient. All liquids were measured and the daily fluid intake was calculated by adding to this amount the estimated water content of the food. In order to insure satisfactory excretion of sugar and nitrogen, the patient was required to drink 2 liters of water each day without regard to thirst.

Urine.—The collection of the 24 hour specimens of urine was accomplished by leaving the urine containers in the patient's room. Care was taken to avoid decomposition. The daily collections were closed and opened at 7.00 a.m. No attempt was made to fractionate the urine into less than 24 hour periods. Bang's method for the quantitative determination of glucose was adopted. The method was controlled from time to time by the use of a standard solution of pure dextrose. Nitrogen was determined by the Kjeldahl method. The total acetone bodies were determined by Van Slyke's (38) method and expressed as acetone. Ammonia was determined by Folin's (15) method; the values were expressed both as grams of ammonia nitrogen and as cubic centi-

meters of tenth normal ammonium hydroxide. The acidity titrable to phenolphthalein was obtained in terms of cubic centimeters of tenth normal acid and this figure was added to the cubic centimeters of tenth normal ammonium hydroxide to determine the total acidity. The hydrogen ion concentration of the urine was estimated by comparison with standard mixtures of buffer salts. Chlorides were determined after Volhard, phosphates by a standard uranium acetate method, and creatinine after Folin. Dextrose to nitrogen (D:N) ratios were calculated for each day by subtracting the carbohydrate content of the ingested food from the glucose value of the urine and dividing the remainder by the total nitrogen of the urine. The daily sugar utilization was calculated by multiplying the number of grams of nitrogen excreted by 3.65, assuming that 58 per cent of the protein metabolized is converted to glucose, adding to the product the number of grams of carbohydrate in the diet, and subtracting the number of grams of glucose excreted. The calculation of the sugar utilization was made for each day of the entire experiment and a sugar tolerance curve was constructed (Chart 1). This method of estimating sugar utilization is similar to that described by Falta (14).

Blood.—Tungstic acid filtrates of whole blood were prepared and analyzed for sugar, non-protein nitrogen, and urea by Folin's (16) methods. The total acetone bodies of whole blood were determined by the Van Slyke-Fitz (39) method; their values were expressed as acetone. The fats of the whole blood were determined by Bloor's method (11). The blood plasma was analyzed for its carbon dioxide-combining power according to Van Slyke's (37) method, and for chlorides by Wetmore's (40) method.

Feces.—Analyses for fat were made in moist feces by Saxon's (32) method. The feces were not studied with the same accuracy as the blood and urine. The bowel movements were all formed and microscopic examination made frequently did not reveal abnormal amounts of fat. After April 21, the feces were collected for a period of 14 days, weighed, and analyzed for fat. A fat excretion of between 5 and 6 per cent of the fat of the food was found fairly constantly. The available fat portion of all diets during the entire period of observation was, therefore, recalculated on this basis to allow for this error.

Respiratory Metabolism.—The metabolic rate was determined

by the gasometric method with analysis of the expired air according to the technique described by Boothby and Sandiford (12).   As determinations of basal metabolism were made in the morning before breakfast the non-protein respiratory quotient was calculated by using the data from urinalysis of the preceding day; the calculation was made in the customary manner according to Lusk's method (24).   The amount of acetone bodies present at any time in the urine was too small directly to affect the calculation of the non-protein respiratory quotient.   At first we had no intention of making an exact study of the respiratory metabolism in this patient so that the tests up to April 30 were carried out as routine determinations; after this date special precautions were adopted to insure the greatest possible accuracy and we have no reason to question the experimental accuracy of any of the results subsequent to that date except the respiratory quotient of May 23 and the basal metabolic rate of May 16.

Case History.

Case A336236, Bessie B., age 28 years, first came to the Mayo Clinic Oct. 4, 1920. She was admitted to the hospital Oct. 6, 1920, dismissed Nov. 19, 1920, readmitted Mar. 29, 1921, and dismissed June 13, 1921.

No familial predisposition to diabetes could be elicited by a careful inquiry among relatives. The patient had attended school regularly from the age of 6 to the age of 16, but was never as rugged as her brothers and sisters. She had never had any infection except two attacks of tonsillitis in childhood. Since the age of 21 years (1913 to 1918) she had weighed 115 pounds and felt perfectly well.

The diabetes was of the severe acute type with abrupt onset dated exactly to the last week in Oct., 1918. The first symptom was a sense of fullness in the stomach and drowsiness, and a few days later, an excessive amount of urine was passed during the 24 hours. Excessive thirst and hunger commenced. A physician was consulted and glycosuria was discovered. A diet limited in sweets and bread resulted in symptomatic improvement.

In November, about 1 month after the onset of diabetes the patient had influenza and was sick for 2 weeks. Recovering from this she continued her diet with only moderate loss of weight and strength until about Jan., 1920, when she began to lose weight more rapidly and was troubled continuously with polyuria and polydipsia. Edema and transient visual disturbances occurred.

Oct. 6, 1920, the patient weighed 36 kilos and measured 157.6 cm., standing height. She was drowsy. Respirations were deep, twenty to the minute, and the breath had a strong acetone odor. Eye-grounds were

normal, and with the exception of a slight haziness, there was no definite cloudiness in the mediums. Examinations of the heart, lungs, and so forth, were negative. Knee jerks were absent; the Wassermann reaction was negative; hemoglobin was 76 per cent; the erythrocytes numbered 4,100,000; leucocytes 6,800; the blood sugar was 0.57 per cent; and carbon dioxide-combining power of the plasma 30 volumes per cent. The first 12 hour specimen of urine measured 2,300 cc., contained 7.7 per cent of sugar, and gave a heavy ferric chloride reaction. The urine was free from albumin and the renal function, measured by blood urea and phenolsulfonephthalein excretion, was normal. The examination in all other respects was negative.

Fasting was instituted Oct. 7 and continued, with a 7 day interval of low protein feeding, until Oct. 23, without freeing the urine of sugar. A second fast between Oct. 27 and 30 cleared the urine, however, and by this time the blood sugar had fallen from 0.57 to 0.13 per cent. During this period of prolonged undernutrition edema developed, with a gain in weight of 24 pounds. The edema disappeared on withholding sodium chloride. The food allowance was raised gradually and Nov. 19, the patient was dismissed from observation on a diet of 20 gm. of carbohydrate, 50 gm. of protein, and 90 gm. of fat, a total of 1,120 calories, with a blood sugar of 0.18 per cent and the urine free from sugar and acetone. Basal metabolic rates of −28 and −32 per cent were observed Nov. 16 and 17. The essential data obtained at that time will be found in Chart 1 of a previous publication (42).

From Nov. 19, 1920 to Mar. 29, 1921, the patient lived at her home in a near-by town, adhering very faithfully to her diet and reporting from time to time. Her weight on leaving the hospital was 35 kilos and she maintained this weight until March, when without any particular reason sugar appeared in the urine and persisted in spite of occasional fast days. She returned to the clinic Mar. 29, weighing 31.6 kilos. Examination did not reveal changes except that a central lens opacity of the granular type was noted in both eyes. Her breath had a faint acetone odor, but respirations were normal and she said she felt well. No infections were found. There were no devitalized teeth and the tonsils were small and apparently not diseased. For the next 11 weeks the patient remained in the hospital and daily examinations were conducted, which will be described. Her general strength and well being remained unimpaired except during the two periods of high protein and high fat diets which provoked a D:N ratio of 3.65 and marked acidosis. Slight drowsiness and hyperpnea occurred in both these periods, but disappeared promptly on discontinuance of this diet, and the patient left the hospital June 13 in a satisfactory condition free from acidosis, and urine free from sugar. The tolerance for glucose, calculating as glucose all the carbohydrate and 58 per cent of the protein metabolized, was apparently as high at the time of her second dismissal as it was on her second admission.

The patient's subsequent course at home was satisfactory for 3 months, then became less favorable, and she died in coma Oct. 3, 2 years and 11 months after the onset of the disease. Necropsy was not obtained.

Summary of the Metabolism Experiments.

The patient proved an ideal subject for metabolism studies. During the long periods of observation she did not fail to cooperate to the fullest extent in the investigation. Earnestly desirous of improving her condition, she maintained perfect serenity throughout her stay in the hospital. The elements of nervous irritability and restlessness which often vitiate metabolism investigations of patients with severe diabetes were, therefore, avoided. Finally, it was a matter of satisfaction that the diabetes was uncomplicated by any infection during these observations.

The experiments were conducted from March 31 to June 13, 1921. The observations are classified in eleven periods:

Period I, 18 days; preliminary observation on a diet of 19.9 gm. of carbohydrate, 48.8 gm. of protein, and 85.5 gm. of fat.

Period II, 2 days of fast followed by 1 day of the diet in Period I.

Period III, 5 days on a diet of carbohydrate 0.7, protein 46.9, and fat 88.3 gm.

Period IV, 4 days on a diet of carbohydrate 1.8, protein 94.2, and fat 99.1 gm.

Period V, 5 days on a diet of carbohydrate 3.3, protein 103.6, and fat 137.9 gm. During Periods IV and V the sugar tolerance failed and in Period V a D:N ratio of 3.65 was observed.

Period VI, 10 days; low protein, relatively high fat, Newburgh type of diet, with recovery of the former tolerance and control of acidosis.

Period VII, 4 days on a diet similar to that in Period VI but somewhat increased.

Period VIII, 8 days; low protein, high fat diet, isocaloric and nearly equal in sugar and ketogenic value with the diet of Period V.

Period IX, 2 days fast followed by 1 day on a low diet.

Period X, 4 days; high protein, high fat diet nearly isocaloric with that of Period VIII, the sugar and ketogenic values equalling those of Period VIII. This diet caused a complete break in sugar tolerance indicated by a D:N ratio of 3.65.

Period XI, 10 days; low protein, relatively high fat diet on which the former sugar tolerance of 30 gm. was regained.

The more important daily data obtained in these experiments are plotted on Chart 1 and recorded in Table I.

Preliminary Observations; 21 Days, Periods I and II.

Period I.—From Mar. 31 to Apr. 17, her home diet of approximately 20 gm. of carbohydrate, 50 gm. of protein, and 90 gm. of fat was continued.

CHART 1.

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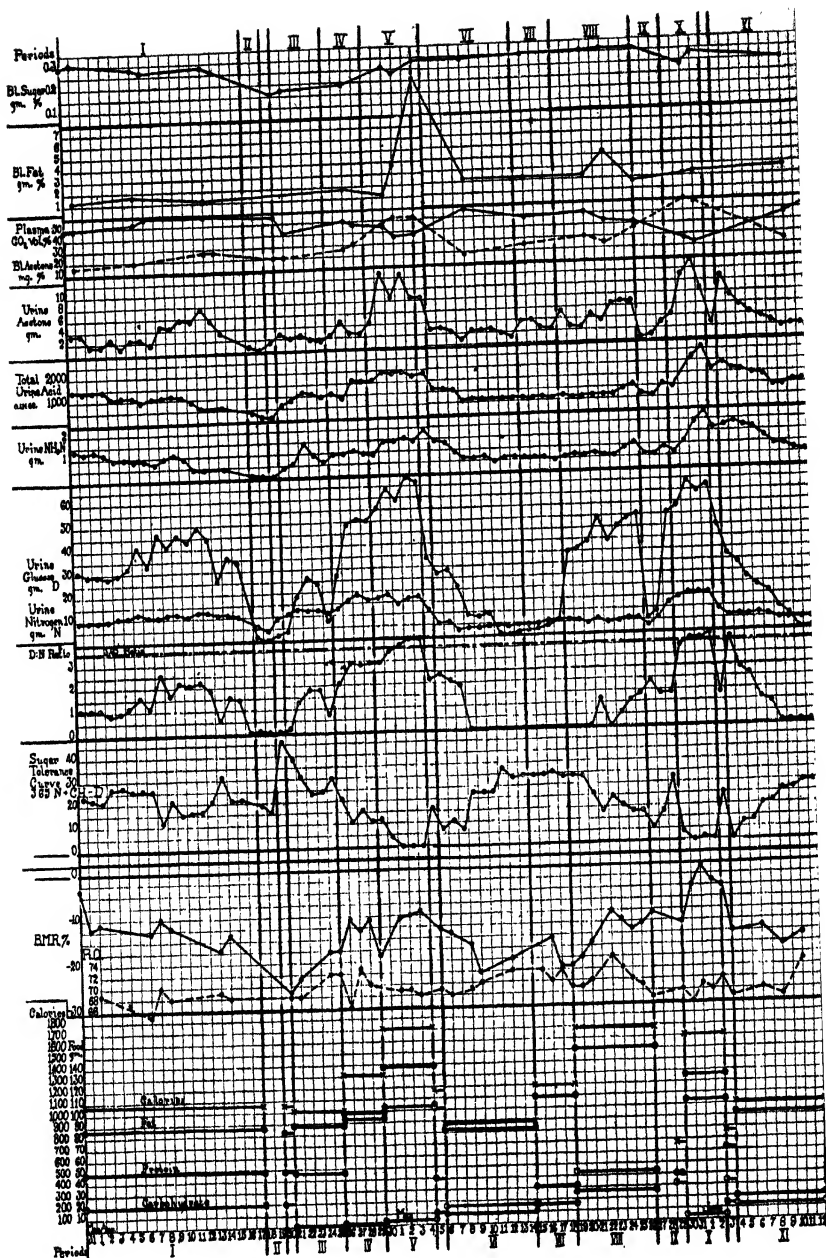
TABLE I.

Metabolism Data in a Case of Diabetes.

Date. 1921	Food intake. Carbohydrate. gm.	Protein. gm.	Fat. gm.	Total calories.	24 hour specimen of urine. Nitrogen. gm.	Glucose. gm.	D:N ratio.	Acetone bodies as acetone. gm.	Ammonia nitrogen. gm.	Phosphates as phosphorus. gm.	Nitrogen balance. gm.	Blood. Sugar per 100 cc. gm.	Acetone bodies as acetone per 100 cc. gm.	Fat per 100 cc. gm.	Carbon dioxide. vol. per cent	Respiratory metabolism. Total calories for each hour.	Basal metabolic rate (Du Bois standard). per cent	Respiratory quotient.	Non-protein respiratory quotient.	Weight. kg.	Period.
Mar. 31	19.9	48.8	85.5	1,077	9.29	30.55	1.15	3.20	1.32	0.805	−1.48	0.294			47.0	43.2	− 4	0.66	0.63	31.5	1
Apr. 1	19.9	48.8	85.5	1,077	8.76	29.53	1.10	3.38	1.26	0.841	−0.95	0.303	0.015	1.23	49.4	39.8	−12	0.68	0.65	31.5	
" 2	19.9	48.8	85.5	1,077	8.71	29.45	1.10	1.65	1.29	0.899	−0.90					40.1	−11	0.69	0.66	31.7	
" 3	19.9	48.8	85.5	1,077	9.82	28.40	0.87	1.56	1.18	0.911	−2.01										
" 4	19.9	48.8	85.5	1,077	10.05	29.05	0.91	2.45	0.96	0.927	−2.24										
" 5	19.9	48.8	85.5	1,077	10.36	32.40	1.21	2.15	0.95	0.86	−2.55										
" 6	19.9	48.8	85.5	1,077	13.22	41.55	1.64	1.20	0.89	1.17	−5.41										
" 7	19.9	48.8	85.5	1,077	10.65	33.10	1.24	2.05	0.82	0.92	−2.84	0.277	0.019	1.50	54.1	39.7	−13	0.65	0.61	32.7	
" 8	19.9	48.8	85.5	1,077	10.77	47.30	2.54	1.84	0.74	1.05	−2.96	0.273			57.5	40.7	−10	0.70	0.67	32.5	
" 9	19.9	48.8	85.5	1,077	11.65	40.48	1.77	4.48	0.94	1.01	−3.84					40.1	−12	0.68	0.67	32.6	
" 10	19.9	48.8	85.5	1,077	11.35	46.25	2.32	4.11	1.04	1.09	−3.54										
" 11	19.9	48.8	85.5	1,077	10.95	43.60	2.16	5.87	0.95	1.04	−3.14										
" 12	19.9	48.8	85.5	1,077	12.74	49.45	2.32	5.72	0.40	0.83	−4.93										
" 13	19.9	48.8	85.5	1,077	12.80	44.74	1.94	7.73	0.38	1.15	−4.99										
" 14	19.9	48.8	85.5	1,077	11.04	26.60	0.61	5.77		1.03	−3.23	0.277	0.026	1.25	58.5	38.2	−17	0.69	0.67	33.6	
" 15	19.9	48.8	85.5	1,077	10.52	36.20	1.55	3.38	0.45	0.99	−2.71	0.266	0.026			39.9	−14	0.68	0.64	33.9	
" 16	19.9	48.8	85.5	1,077	10.00	33.90	1.40				−2.19										
" 17	19.9	48.8	85.5	1,077	10.74	37.00	1.59				−2.93										

II	Apr. 18	0	0	0	0	5.68	1.25	0.22	0.95	0.17		−5.68									
	" 19	0	0	0	0	4.43			0.29	0.13	0.43	−4.43									
	" 20	19.9	48.8	84.6	1,069	8.08	2.26	1.58	1.58	0.08	0.80	−0.27									
III	Apr. 21	0.7	46.9	88.3	1,016	11.54	4.00	0.29	2.85	0.37	1.03	−4.04	0.166	0.020		56.6	−27	34.1	0.68	0.64	34.1
	" 22	0.7	46.9	88.3	1,016	13.43	18.90	1.36	2.00	0.53	0.98	−5.93	0.178	0.020		40.9	−24	34.7	0.68	0.62	33.3
	" 23	0.7	46.9	88.3	1,016	13.27	25.50	1.88	2.33	1.43	1.03	−5.77									
	" 24	0.7	46.9	88.3	1,016	13.25	24.66	1.81	1.90	0.95	1.12	−5.75									
	" 25	0.7	46.9	88.3	1,016	10.43	8.77	0.77	1.39	0.65	1.01	−2.93					−18	37.0	0.72	0.71	32.1
IV	Apr. 26	1.8	94.2	99.1	1,315	13.00	27.88	2.01	2.26	0.82	1.20	+2.07					−18	37.0	0.72	0.69	31.7
	" 27	1.8	94.2	99.1	1,315	16.00	49.40	2.98	4.82	0.90	1.26	−0.93					−11	40.1	0.66	0.63	31.6
	" 28	1.8	94.2	99.1	1,315	18.06	51.44	2.75	2.89	1.14	1.37	−2.99	0.197	0.026	1.71	50.4	−13	39.5	0.73	0.74	32.0
	" 29	1.8	94.2	99.1	1,315	16.36	50.20	2.96	2.39	0.99	1.40	−1.29		0.030		47.0	−11	40.3	0.70	0.70	32.1
V	Apr. 30	3.3	103.6	137.9	1,721	17.55	55.30	2.96	4.13	0.88	1.50	−0.97					−19	36.9	0.71	0.73	31.8
	May 1	3.3	103.6	137.9	1,721	17.86	64.20	3.41	12.85	1.40	1.69	−1.28					−11	39.8	0.69	0.70	31.7
	" 2	3.3	103.6	137.9	1,721	14.83	59.08	3.76	8.62	1.44	1.40	+1.75	0.256	0.049	1.31	45.2	−10	40.8	0.69	0.71	31.7
	" 3	3.3	103.6	137.9	1,721	16.55	69.20	3.98	12.50	1.52	1.36	+0.03	0.235	0.053	3.80	36.9	−9	41.4	0.68	0.70	31.9
	" 4	3.3	103.6	137.9	1,721	17.00	67.55	3.78	8.85	1.44	1.08	−0.42									
VI	May 5	9.6	39.4	103.3	1,162	11.09	34.15	2.21	8.47	1.88	0.85	−4.79	0.282	0.053	11.20	38.6	−13	39.2	0.69	0.68	31.0
	" 6	15.6	9.9	83.4	881	5.14	27.14	2.25	3.00	1.33	0.57	−3.56					−14	38.7	0.68	0.68	31.5
	" 7	15.6	9.9	83.4	881	6.50	28.30	1.95	3.00	1.27	0.50	−4.92									
	" 8	15.6	9.9	83.4	881	3.70	21.32	1.55	2.51	0.82	0.57	−2.12									
	" 9	15.6	9.9	83.4	881	4.35	9.25		0.97	0.62	0.45	−2.77					−17	37.2	0.69	0.68	31.8
	" 10	15.6	9.9	83.4	881	4.43	8.63		2.32	0.54	0.47	−2.85	0.285	0.019	2.38	58.9	−23	35.1	0.70	0.68	32.3
	" 11	15.6	9.9	83.4	881	4.60	9.30		2.20	0.71	0.55	−3.02									
	" 12	15.6	9.9	83.4	881	4.84			2.39	0.47	0.49	−3.26									
	" 13	15.6	9.9	83.4	881	3.55			1.91	0.56	0.37	−1.97									
	" 14	15.6	9.9	83.4	881	4.25			0.96	0.57	0.47	−2.67					−20	36.0	0.72	0.70	31.7

TABLE I—*Concluded.*

Date (1921)	Food intake — Carbohydrate (gm)	Protein (gm)	Fat (gm)	Total calories	24 hour specimen of urine — Nitrogen (gm)	Glucose (gm)	D : N ratio	Acetone bodies as acetone (gm)	Ammonia nitrogen (gm)	Phosphates as phosphorus (gm)	Nitrogen balance (gm)	Blood — Sugar per 100 cc (gm)	Acetone bodies as acetone per 100 cc (gm)	Fat per 100 cc (gm)	Carbon dioxide (vol per cent)	Respiratory metabolism — Total calories for each hour	Basal metabolic rate (Du Bois standard) (per cent)	Respiratory quotient	Non-protein respiratory quotient	Weight (kg)	Period
May 15	14.7	30.7	109.1	1,201	4.78			3.88	0.59	0.40	+0.13	0.300	0.026	2.30	49.0	30.5	−33?	0.72	0.70	31.2	VII
" 16	14.7	30.7	109.1	1,201	4.83			3.97	0.55	0.46	+0.08					37.8	−16	0.70	0.69	31.2	
" 17	14.7	30.7	109.1	1,201	5.49			2.57	0.50	0.49	−0.58					35.3	−22	0.72	0.70	31.3	
" 18	14.7	30.7	109.1	1,201	5.45			2.12	0.49	0.56	−0.54										
May 19	42.6	26.5	151.4	1,692	5.72	35.00		5.27	0.55	0.55	−1.48					35.2	−22	0.69	0.67	31.0	VIII
" 20	42.6	26.5	151.4	1,692	5.11	35.20		2.96	0.60	0.41	−0.87					35.9	−20	0.69	0.66	30.5	
" 21	42.6	26.5	151.4	1,692	4.40	38.10		2.37	0.56	0.38	−0.16					37.3	−17	0.70	0.67	30.8	
" 22	42.6	26.5	151.4	1,692	5.80	49.90	1.26	4.40	0.63	0.56	−1.56	0.303	0.030	2.40	50.4	40.3	−10	0.74	0.74	30.5	
" 23	42.6	26.5	151.4	1,692	4.61	39.90		3.46	0.58	0.45	−0.37					39.2	−12	0.72	0.71	30.6	
" 24	42.6	26.5	151.4	1,692	4.96	45.30	0.54	6.22	0.61	0.49	−0.72	0.312	0.025	4.14	44.7	38.3	−14	0.70	0.68	30.4	
" 25	42.6	26.5	151.4	1,692	5.24	48.50	1.13	6.54	0.82	0.54	−1.00					38.9	−13	0.69	0.68	30.5	
" 26	42.6	26.5	151.4	1,692	5.76	50.40	1.35	6.54	1.11	0.58	−1.52										

Date																					Period
May 27	0	0	0	0	3.45	6.50	1.88	0.61	0.67	0.16	−3.45	0.317	0.038	1.64	40.7	39.3	−11	0.67	0.66	30.3	IX
" 28	0	0	0	0	5.83	7.72	1.32	0.77	0.58	0.29	−5.83										
" 29	33.0	40.0	39.5	666	12.28	50.10	1.39	2.90	0.82	0.87	−5.88										
May 30	3.8	104.8	126.3	1,621	14.75	53.85	3.39	4.02	0.62	1.02	+2.02					39.0	−13	0.68	0.64	31.3	X
" 31	3.8	104.8	126.3	1,621	16.43	64.30	3.68	10.75	1.08	1.18	+0.34					42.7	−5	0.66	0.66	31.7	
June 1	3.8	104.8	126.3	1,621	15.40	59.60	3.62	13.52	1.86	1.47	+1.37	0.240	0.060	2.07	26.8	44.4	−1	0.69	0.71	31.6	
" 2	3.8	104.8	126.3	1,621	15.40	63.00	3.84	8.54	2.44	1.00	+1.37	0.285	0.059	2.30	23.8	42.8	−4	0.68	0.69	30.4	
June 3	33.6	14.0	62.0	772	8.58	44.90	1.32	2.69	1.67	0.75	−6.34					42.7	−5	0.70	0.73	30.4	XI
" 4	12.3	19.7	94.1	1,006	5.76	33.10	3.61	10.40	1.79		−2.61					37.9	−15	0.67	0.65	30.6	
" 5	12.3	19.7	94.1	1,006	6.27	27.79	2.47	7.28	1.80	0.64	−3.12										
" 6	12.3	19.7	94.1	1,006	5.29	23.88	2.19	5.72	1.63	0.56	−2.14										
" 7	12.3	19.7	94.1	1,006	6.00	18.92	1.10	4.02	1.55	0.56	−2.85					38.6	−14	0.68	0.68	31.2	
" 8	12.3	19.7	94.1	1,006	5.54	16.80	0.81	3.50	1.34	0.50	−2.39										
" 9	12.3	19.7	94.1	1,006	4.94	9.08		2.81	0.89	0.44	−1.79					36.9	−18	0.67	0.65	31.7	
" 10	12.3	19.7	94.1	1,006	4.60	6.78		1.93	0.80	0.48	−1.45										
" 11	12.3	19.7	94.1	1,006	4.62			2.95	0.72	0.48	−1.47	0.250	0.024	2.75	46.6	37.9	−16	0.73	0.71	31.7	
" 12	12.3	19.7	94.1	1,006	4.60			2.69	0.62		−1.45										

Height (standing) = 157.6 cm.; height (sitting) = 82.5 cm.; chest = 71.5 cm.; age = 29 years.

As an average of 6 per cent of the fat in the food was found in the fēces, this allowance has been made in the tabulation of the fat intake.

In addition to the data tabulated above, the following were obtained but have not been incorporated in the table for lack of space: water balance, ordinary urine examinations, the titratable acidity, the hydrogen ion concentration, the creatinine, and the chlorides of the urine; the plasma chlorides, blood urea, blood creatinine, and blood non-protein nitrogen.

In calculating the non-protein respiratory quotient the data from urinalysis of the previous day were used.

In using the tables to determine the influence of food on the basal respiratory metabolism it must be remembered that the tests were not influenced by the food recorded for that day and that a metabolism test on the first morning of a new period really belongs to the preceding period; for example, the respiratory data of June 3 must be interpreted with the urinary data of June 2 and, therefore, belongs to Period X although recorded with Period XI.

In the calculation of the nitrogen balance no allowance was made for fecal nitrogen.

During this time an average daily excretion of 37 gm. of glucose and 10.74 gm. of nitrogen occurred. The D:N ratio fluctuated somewhat, averaging 1.52. The blood sugar was 0.294 per cent on admission and a slight acidosis was indicated by the ammonia nitrogen excretion which exceeded 1 gm. and by total acetone bodies of over 3 gm. in the 24 hour specimen of urine. The carbon dioxide-combining power of the plasma was 47.0 volumes per cent on admission. A daily administration of 15 gm. of sodium bicarbonate was commenced Apr. 11, but discontinued Apr. 20. Thereafter no drugs were used. The average basal metabolic rate for the period was −12 per cent, the average respiratory quotient was 0.68, and the non-protein respiratory quotient, 0.65. The morning of Apr. 7, the very questionable non-protein respiratory quotient of 0.61 was obtained.

The utilization of glucose from the carbohydrate of the diet and from protein metabolism averaged 24.6 gm. for the first 6 days, but after that, fell to an average of 21.2 gm. for the rest of the period.

Period II.—Apr. 18 and 19 the patient was fasted. Apr. 20 she again received the food given in Period I. The beneficial effect of the 2 fast days confirmed the general belief in the value of an occasional fast day in the management of diabetes. Apr. 19, the urine was free from sugar.

High Protein, High Fat Diets with Development of a D:N Ratio 3.65; 14 Days, Periods III, IV, and V.

Period III.—From Apr. 21 to 25, the patient was placed on a protein-fat diet of meat, butter, eggs, and soy bean bread, containing less than 1 gm. of carbohydrate, 46 9 gm. of protein, and 88.3 gm. of fat. This was continued 5 days without much appreciable effect. The carbon dioxide-combining power of the plasma remained normal. The D:N ratio did not exceed 1.88. The basal metabolic rate fell to −27 per cent Apr. 21, the result probably of the 2 fast days, and had risen to −18 per cent by Apr. 25.

Period IV.—From Apr. 26 to 29, the protein allowance was increased by approximately 50 gm. to twice the previous amount, the fat of the diet remaining about the same as before, and the carbohydrate not exceeding 2 gm. The effect of this change was evident immediately. The urine of the first day showed a D:N ratio of 2.01. Apr. 27, 28, and 29, the ratios were 2.98, 2.75, and 2.96. Acidosis, however, was still slight and the patient felt entirely comfortable. The total acetone bodies in the urine of Apr. 29, were 2.39 gm., that is, not higher than the acetone excretion during the preliminary period; the carbon dioxide-combining power of the plasma remained normal, 47.0 volumes per cent, and the ammonia nitrogen excretion averaged less than 1 gm.

The pernicious influence of this added protein on sugar utilization is very striking. Unless we assume a flooding out of stored glycogen, which seems unlikely in view of the long preceding period of very low carbohydrate intake, or unless nitrogen retention had occurred, for which there is no evidence, the rising D:N ratios indicate a sharp drop in sugar toler-

ance. This break in tolerance cannot be attributed to increased acidosis or ketogenesis since neither of these increased over that of Period I. Another significant result of raising the protein may be the elevation that takes place in the basal metabolic rate. This increases from an average of −20 per cent for three determinations between Apr. 22 and 26 to an average of −14 per cent for four determinations between Apr. 27 and 30. This is likewise unexplained by acidosis and seems to be attributable to a prolonged cumulative effect of the specific dynamic action of the extra protein (25, 26, a).

Period V.—From Apr. 30 to May 4, the fat in the diet was increased by 39 gm., and the protein by 10 gm., the carbohydrate quota remaining practically the same. The result of this was a rapid loss of the remaining ability to utilize sugar and the excretion of glucose in amounts equal to the maximal theoretic yield of glucose from the protein metabolized. The D:N ratios obtained in the 24 hour collections of urine of May 1, 2, 3, and 4 were 3.41, 3.76, 3.98, and 3.78, or an average of 3.73. The non-protein respiratory quotients of May 2, 3, and 4 were 0.70, 0.71, and 0.70. The rate of excretion of acetone bodies increased, the blood acetone rose to 0.049 May 2, and 0.053 May 3, and the carbon dioxide-combining power of the plasma fell to 36.9 volumes per cent May 3. The percentage of fat in the blood increased very rapidly. The basal metabolic rate rose only slightly, from the average of −14 per cent of the previous period to an average of −10 per cent for this period. In spite of the increased acidosis and a D:N ratio of 3.73, the elevation of the basal metabolic rate was not, therefore, as great as that caused by the increase of about 50 gm. of protein in the preceding period. The morning of May 5, the patient complained of fatigue and was drowsy and it was considered advisable to change the diet.

Low Protein, Relatively High Fat Diets with Recovery of Former Carbohydrate Tolerance; 14 Days, Periods VI and VII.

Period VI.—Beginning with the noon meal of May 5, and continuing until May 14, the diet was made similar to the therapeutic diets employed by Newburgh and Marsh (29). The daily allowance was 15.6 gm. of carbohydrate, 9.9 gm. of protein, and 83.4 gm. of fat, a total of 881 calories. According to the Du Bois (13, 19) formula the basal calorie requirement for this patient, 158 cm. in height and weighing 31 kilos, would be, 1,070 calories, provided the basal metabolic rate was normal. This, however, fell within a few days after the new diet was instituted and at a basal metabolic rate of −20 per cent, 856 calories suffice for the basal energy exchange. As the patient was kept in bed, the new diet provided, therefore, nearly enough calories to avoid calling on endogenous food supplies. The therapeutic results of the diet on this completely diabetic subject with increasing acidosis and impending coma are very satisfactory, as the daily figures in Table I reveal. Within 7 days the urine became sugar-free and ketogenesis was practically controlled. The ferric chloride

test of the urine was negative within 2 days and the rate of ammonia excretion fell almost equally rapidly. The carbon dioxide-combining power of the plasma reaccumulated to 58.9 volumes per cent by the 5th day and the blood fat was appreciably reduced. The postabsorptive blood sugar value, however, remained elevated. The nitrogen excretion fell off rapidly reaching 3.55 gm. on the 9th day.

The figures for the respiratory metabolism reveal a decrease in the level of the basal metabolic rate to −20 per cent on May 13th. The respiratory quotients remained unchanged and their failure to mirror improvement in carbohydrate utilization may be accounted for in a slight measure by glycogen storage, but in a large measure by the fact that the tests were made in the morning before breakfast, too long after the last feeding to be affected by the small amount of carbohydrate ingested. The rapid control of ketogenesis and acidosis, which the new diet accomplished, is fair evidence that sugar was actually burning. Judged by data from urinalysis the patient's sugar tolerance at the end of the period exceeded that of the preliminary period. The average daily sugar utilization for the 3 days, May 12, 13, and 14, equaled 30 gm.

Period VII.—Between May 15 and 18 the protein allowance was increased to 30.7 gm. and the fat allowance to 109.1 gm. Traces of sugar reappeared in the urine, but otherwise the condition of the patient remained practically unchanged. The glucose utilization for these 4 days averaged 29.7 gm. The increase in protein to 30.7 gm., which is nearly 1 gm. for each kilo of the patient's body weight, had no such cumulative effect on the basal metabolic level as was observed in the higher protein periods, nor did the increase in fat to 109.1 gm. alter the basal metabolic rate which continued −20 per cent.

Low Protein Diet with Fat Sufficient to Make It Isocaloric with the High Protein, High Fat Diet of Period V, and Carbohydrate Sufficient to Yield Available Glucose Equal to That of Period V; 8 Days.

Period VIII.—From May 19 to 26, the diet contained 42.6 gm. of carbohydrate, 26.5 gm. of protein, and 151.4 gm. of fat. Our purpose was to compare the effect of this high fat, low protein combination with that of the former high protein diet of Period V (Apr. 30 to May 4, inclusive). The two diets are practically isocaloric, the former yielding 1,721 calories and the latter 1,692. Both diets should theoretically yield nearly the same amount of glucose. In the former, this was derived from 3.3 gm. of carbohydrate and 58 per cent of 103.6 gm. of protein; the latter contained only 26.5 gm. of protein which could yield only 15.4 gm. of glucose, hence 42.6 gm. of carbohydrate were added to it. The two diets are practically comparable in energy value and available glucose, but different in protein. They may also differ slightly in ketogenic properties by Woodyatt's (45) assumption that 46 per cent by weight of the protein of the diet should be calculated as the equivalent of a like weight of oleic acid in ketogenic properties.

The diet of Period V contained the equivalent of 171.7 gm. of theoretic oleic acid (F. A. = 0.46 P. + 0.9 F.) while that of Period VIII has only 148.5 gm. On this diet, as was to be expected, sugar reappeared promptly in the urine. The amount increased gradually, but did not exceed 50.4 gm. The D:N ratio was 1.26 on the 4th day, 1.13 on the 7th day, and 1.35 on the 8th day. The excretion of acetone bodies increased, but did not exceed 6.54 gm. The ammonia nitrogen excretion remained below 1 gm. a day until the 8th day when it reached 1.11 gm. The carbon dioxide-combining power of the plasma was 50.4 volumes per cent on the 4th day, and 44.7 on the 6th day. The blood acetone value of the 6th day was 0.025 gm. The basal metabolic rate increased from an average of −20 to −12 per cent May 23 to 27. The non-protein respiratory quotients continued to hover close to 0.70 in tests made 14 hours after a meal. Quotients in tests conducted $\frac{1}{2}$, 1, 2, and 3 hours after meals showed a peculiar behavior, to be discussed under respiratory quotients.

Subjectively, the patient continued free from complaints. Pulse and respiration were normal and weight remained constant. It was evident that the diet was tolerated much better than the former isocaloric diet of high protein; while there was a general tendency toward a lower sugar tolerance this was by no means so marked as it had been on the high protein diet, and even on the 8th day there seems to have been a utilization of 13.2 gm. of glucose from protein.

It is impossible to know whether to attribute such effects as were observed to the high fat value of the diet or to the high sugar value. The latter was well above the patient's tolerance of 30 gm. of glucose and it is a generally well established principle in diabetic therapy that the feeding of carbohydrate in excess of sugar tolerance will depress such tolerance as exists. Had the high fat diet been given with a smaller amount of carbohydrate it is possible that it would have been tolerated even better than it was.

Period IX.—2 fast days were instituted at this time, May 27 and 28, and on May 29 the food contained 33 gm. of carbohydrate, 40 gm. of protein, and 39.5 gm. of fat. The purpose of this fast period was to correct the moderate acidosis which had been provoked by the preceding diet and to restore the patient to the favorable condition of the beginning of Period VIII so that the effect of the diet of the following period could be compared with that of Period VIII. The benefit of this fast was less striking than that produced by the 2 fast days which followed Period I, suggesting that the diet of Period VIII had produced not only an immediate deleterious effect, but one from which recovery was slow. Whether to attribute this to the fat or to the carbohydrate is questionable, the observation is like those made by Allen (2) on partially depancreatized dogs fed on fat.

High Protein, High Fat Diet with Ketogenic Value Nearly equal to That of Period VIII; D:N Ratio 3.65; 4 Days.

Period X.—It appeared desirable to compare the effect of the diet of Period VIII with that of a diet which would be exactly comparable to it in ketogenic constituents as calculated by Woodyatt's (45) formula and yet contain a larger proportion of protein. Accordingly a diet was given from May 30 to June 2 which contained 3.8 gm. of carbohydrate, 104.8 gm. of protein, and 126.3 gm. of fat. Inserting these values into the formula (F. A. = 0.46 P. + 0.9 F.) it will be seen that this diet provides the equivalent of 161.8 gm. of oleic acid, which is close enough to the ketogenic value of the diet of Period VIII to satisfy the requirements. The new diet also contained practically the same amount of available glucose (3.8 gm. as carbohydrate and 58 per cent of 104.8 gm. of protein) and differed very little in its amount of available energy.

This second high protein, high fat diet was started May 30 and continued for 4 days. The D:N ratio of the urine of May 29 was 1.39. The data from urinalysis reveal a sugar utilization of 27.7 gm. on this date. The ammonia nitrogen excretion was 0.82 gm. and acetone excretion below 3 gm. The D:N ratio of the urine of the first 24 hours of the new high protein diet jumped to 3.39 and May 31, June 1, and June 2, it was that of complete diabetes, 3.68, 3.62 and 3.84, an average of 3.63 for the 4 days. Accelerated ketogenesis and acidosis developed more slowly than the drop in sugar tolerance. The total acetone bodies of the urine of the 1st day were 4.02 gm.; on the 2nd day they were 10.75 gm., on the 3rd day 13.52 gm. The excretion of ammonia nitrogen was only 0.62 gm. on the 1st day rising to 1.08 gm. on the 2nd day, to 1.86 gm. on the 3rd day, and to 2.44 gm. on the 4th day. The total acetone bodies of the blood rose to 0.060 and 0.059 gm. on the last 2 mornings. On the 3rd morning the carbon dioxide-combining power of the plasma was 26.8 volumes per cent; on the 4th morning it was 23.8. By the 3rd day the breath smelled strongly of acetone and a slight hyperpnea was noticeable. On the 4th day slight nausea was complained of and it was decided that the period should be discontinued.

The respiratory metabolism behaved as it had on the high protein diet of Period V. The basal metabolic rate rose from −13 per cent the morning of May 30, the beginning of the period, to −5 per cent May 31 and −1 per cent June 1. The average rate for the period was −4 per cent. The experimental basal respiratory quotients for the 4 days were 0.66, 0.69, 0.68, and 0.70 and the corresponding calculated non-protein respiratory quotients were 0.66, 0.71, 0.69, and 0.73 (average 0.70). The effect of meals on these quotients is referred to in the discussion of the respiratory quotient.

The effect of the protein on sugar tolerance and ketogenesis appeared to be out of proportion to its ketogenic properties as calculated by Woodyatt's (45) formula. The effect is certainly much more marked than was that of the nearly isocaloric high fat diet of Period VIII which it was designed to equal in ketogenic properties.

Low Protein, Relatively High Fat Diet with Recovery of Former Tolerance; 10 Day Period.

Period XI.—The morning of June 3 a test breakfast of 25 gm. of pure glucose was given (to be discussed under specific dynamic action of foods), but at noon of this day the patient was placed on a diet similar to the Newburgh (29) type of diet of Period VII. It differed merely in detail, being designed to provide 0.66 gm. of protein for each kilo of body weight, fat in sufficient amount for adequate calories, and carbohydrate equal to, but not exceeding, the patient's tolerance as previously determined. Account was taken of a theoretically desirable ketogenic antiketogenic ratio between the various constituents of the food. This diet was determined as follows.

On the basis of the Du Bois standards, a woman aged 29 years, weighing 31.3 kilos, 158 cm. in height, and with a normal metabolic rate would require 1,070 calories to meet her basal requirement. The protein allowance was fixed at 20.7 gm., 0.66 gm. for each kilo of weight. This amount of protein may theoretically yield 12 gm. of glucose (0.58 × 20.7). Assuming the glucose tolerance to be 25 gm., the patient should be able to burn additional carbohydrate to the amount of 13 gm. Fat was required to supply the necessary calories, but to avoid ketogenesis a definite ratio must not be exceeded between the fat of the diet and the available glucose. On the basis of other studies it seems possible that a diet which is constructed so as to provide not more than 1 gm. of protein for each kilo of body weight and calories not exceeding maintenance requirements may, without being ketogenic, contain fat in an amount such that one-half a molecule of glucose will be available to accompany the metabolism of every molecule of fatty acid. A diet of 13 gm. of carbohydrate, 20.7 gm. of protein, and 99.5 gm. of fat would give 1,064 calories, an energy value sufficient for the basal energy exchange of the patient. Since a basal metabolic rate of −15 to −20 per cent was to be anticipated, such a diet would be a maintenance diet without extra allowance for the specific dynamic action of food or for the slight exertion the patient was to be allowed to make. It was hoped that the diet would clear up the glycosuria, control the ketogenesis and acidosis, and maintain the patient in nitrogen and calorie equilibrium.

The subsequent course justified our expectations. The diet actually given, on recalculation contained 12.3 gm. of carbohy-

drate, 19.7 gm. of protein, and 94.1 gm. of fat, totalling 1,006 calories. Sugar disappeared from the urine in 8 days and the ferric chloride reaction was negative on the 6th day. The carbon dioxide-combining power of the plasma reached 46.6 volumes per cent on the 9th day and 54.1 volumes per cent on the 11th day. The data from urinalysis revealed a glucose utilization of 29 gm. on each of the last 2 days before dismissal. A satisfactory nitrogen balance was not obtained; a daily negative balance of 1.5 gm. occurred on each of the last 2 days. It seemed possible, however, that with time this would adjust itself, and in any case, the patient was unable to remain longer in the hospital. She was dismissed, therefore, on this diet, weighing 31.7 kilos. She left Rochester and returned to her home in a near-by town reporting to us weekly by telephone. For 3 months she maintained her weight and the urine remained free from sugar and with a negative ferric chloride reaction. Sugar then reappeared in the urine and the patient becoming discouraged discontinued her diet and died in coma soon after. In view of the low tolerance, the very narrow dietary margin of safety, the type of the disease, and its duration, it is unlikely that disaster could have been prevented for long under any circumstances.

The Respiratory Quotient.

The average respiratory quotients for the various dietetic periods are as follows:

Period.	Number of tests.	Respiratory quotient.	Non-protein respiratory quotient.
I	8	0.68	0.65
II	1	0.68	0.64
III	3	0.71	0.67
IV	4	0.70	0.70
V	3	0.69	0.70
VI	5	0.70	0.68
VII	4	0.71	0.69
VIII	7	0.70	0.69
IX	1	0.68	0.64
X	4	0.68	0.70
XI	4	0.69	0.67
Average.....	44	0.693	0.679

Excepting some of the respiratory quotients obtained in Periods I and III, we feel quite sure of the technical accuracy of the figures. The fact that they vary only slightly above and below the quotient for fat (0.707) indicates, we believe, that at the time of their determination no carbohydrate and only a small amount of protein was being burned. The variation from 0.71 was probably due to the following factors: (a) temporary alteration in the character of the respirations so common in all respiration experiments; (b) variation in the carbon dioxide-combining power of the blood depending on whether the acidosis is increasing or diminishing at the time of the test; and (c) utilization of oxygen and carbon dioxide in the formation of acetone bodies. As pointed out by Lusk (24) it is impossible to make correct allowances for these factors which influence the respiratory quotient and, therefore, we have not attempted such calculation. In consequence of the possible magnitude of the first of these factors we believe that the occasional respiratory quotients below 0.71 cannot be taken to indicate the formation of sugar from fat, particularly, since such deviation is neither marked nor constant; furthermore, the other data of this study are contrary to any such assumption. In this connection the five experiments performed under postabsorptive standard conditions May 27, give the range of variation caused by these factors the most important of which, in this instance was probably the unconscious alteration in the character of the respiration: 0.67, 0.69, 0.68, 0.71, and 0.72. The effect of fright is reflected in the high respiratory quotient, 0.74, of May 23 which without question was caused by slightly forced breathing following the dropping of a tray of dishes beside the patient's door while the test was in progress. Our patient was most willing and cooperated in every way; we fear that possibly she tried overhard to be quiet and breathe normally and as a result at times during the collection of the expired air slightly underventilated her lungs which resulted in decreased elimination of carbon dioxide and a consequent lowering of the respiratory quotient.

The non-protein respiratory quotient averages, calculated by Lusk's (24) method, are only slightly different from those found experimentally. It is doubtful if they are a more exact approximation of the average 24 hour combustion quotients for fat and carbohydrate because they are calculated on the assumption that

the relation between the protein, fat, and carbohydrate metabolism remains constant for the entire day; this assumption is probably not justified. Likewise, Shaffer (35) has shown that no more accurate results are obtained by a method of calculation which attempts to allow separately for both the increase in the respiratory quotient from the glucose quota of the protein and the opposite ketogenic effect of protein; he advises, therefore, the use of the total respiratory quotient for the analysis of experimental data. Shaffer has constructed a very interesting and valuable table by which the ratio of the ketogenic to antiketogenic molecules in the metabolic mixture may be determined from the respiratory quotient and also the percentage of calories obtained from glucose and fatty acid; the latter is an elaboration of Lusk's (26, a) modification of the table of Zuntz and Schumburg. This method, however, is not strictly applicable to respiratory quotients obtained as ours, for short basal morning respiratory periods with the patient taking food subsequent to the test, and should probably be reserved for respiration experiments conducted in a respiration calorimeter for periods of 24 hours when the combined influence of rest, work, and food can be obtained in the day's average and where the effect of temporary alterations in the character of the respiration do not play a part in the elimination of carbon dioxide.

In spite of these theoretic objections to the submission of the data on Bessie B. to an analysis by Shaffer's calculations, it must be admitted that the results are noticeably consistent therewith except in regard to the intensity of the acidosis that would be expected if it depended primarily on the magnitude of the ketogenic antiketogenic ratio as calculated by his method from the respiratory quotients.

The Specific Dynamic Action of Food and the Behavior of the Respiratory Quotient Following the Ingestion of Food.

Nine experiments were made in order to study the specific dynamic action of food and its effect on the respiratory quotient. The results are listed in Table II. They can be conveniently divided into three series corresponding to diets of Periods VIII, X, and XI. Each experiment consisted of a series of five determinations of the metabolic rate and respiratory quotient. The first

The Specific Dynamic Action of Test Meals and Their Effect on the Respiratory Quotient.*

Bessie B. (A336236)

1921	Series I (Period VIII).					Series II (Period X).			Series III (Period XI).
	May 23	May 24	May 25	May 26	May 27	June 1	June 2	June 3	June 11
D:N ratio (preceding day)	1.26	—	0.54	1.13	1.35	3.68	3.62	3.84	—
D:N " (day of observation)	—	0.54	1.13	1.35	1.88	3.62	3.84	1.32	—
Basal metabolic rate, per cent	-10	-12	-14	-13	-11	- 1	- 4	-5	-16
Respiratory quotient	0.74	0.72	0.70	0.69	0.67	0.69	0.68	0.70	0.73
Test breakfast:									
Protein, gm	8.8	8.8	8.8	8.8	Control ex-	34.9	34.9		6.5
Fat, gm	50.5	50.5	50.5	50.5	periment,	42.1	42.1		31.4
Carbohydrate, gm	14.2	14.2	14.2	14.2	no food.	1.3	1.3	25 (glucose)	4.1
Later observations of metabolic rate.									
½ hour {Metabolic rate	+14	+ 4	+ 7	+ 6	-15	+ 8	+14	0	- 2
Respiratory quotient	0.69	0.69	0.70	0.66	0.69	0.61	0.62	0.69	0.68
1 " {Metabolic rate	+ 8	- 1	+ 8	+ 6	-10	+16	+ 5	+3	+ 1
Respiratory quotient	0.70	0.70	0.70	0.68	0.68	0.63	0.65	0.70	0.67
2 hours {Metabolic rate	+16	+ 4	+14	+ 5	- 8	+20	+24	-6	- 5
Respiratory quotient	0.71	0.70	0.70	0.67	0.71	0.65	0.65	0.67	0.70
3 " {Metabolic rate	+10	+ 1	+ 9	+ 5	-12	+14	+26	-9	- 7
Respiratory quotient	0.69	0.69	0.70	0.69	0.72	0.64	0.66	0.67	0.67

*In using the tables to determine the influence of food on the metabolic rate it must be remembered that the basal tests were not influenced by the food recorded for that day and, therefore, must be considered in the light of the previous day's diet and D:N ratio; however, the subsequent observations after the ingestion of the test meal will

determination immediately preceded a test meal; the other four followed $\frac{1}{2}$, 1, 2, and 3 hours after the meal.

The first series of experiments, five in number, the last of which was a control without food, were made May 23, 24, 25, 26, and 27, during Period VIII when the diet of the day preceding each observation consisted of 26.5 gm. of protein, 151.4 gm. of fat, and 42.6 gm. of carbohydrate. During this time the D:N ratio varied as follows: 1.26, 0, 0.54, 1.13, and 1.35; and, May 23, when glucose utilization was greatest, the calculated combustion of glucose from all sources did not exceed 20 gm. Therefore, these experiments, with the exception of those on the 2nd day, were made on an organism which presumably was not utilizing carbohydrate as such and was metabolizing only a part of the sugar derived from protein. The test meal given, protein 8.8 gm., fat 50.5 gm., carbohydrate 14.2 gm., was one-third of the total daily food allowance. In view of the pure glucose experiment of June 3, it is unlikely that the carbohydrate quota of this meal played any material part in the reaction produced; unfortunately no determinations on the effect of protein or fat alone were obtained and it is, therefore, impossible to assign exactly the part each of these played in the reaction. A rough estimate of the relative rôle of protein and fat can, however, be gathered from the experiments of the second series carried out in Period X in which the protein in the test meal was increased to 34.9 gm. and the fat decreased to 42.1 gm.

The second series of experiments was conducted in Period X, June 1, 2, and 3. The diet of the day preceding each observation consisted of 104.8 gm. of protein, 126.3 gm. of fat, and 3.8 gm. of carbohydrate. From the D:N ratios of 3.68, 3.62, and 3.84 it is assumed that the patient was completely diabetic and burning neither the carbohydrate of the food nor the sugar derived from the protein. The test meal in two of these experiments was protein 34.9 gm., fat 42.1 gm., and carbohydrate 1.3 gm. In the third experiment 25 gm. of glucose were given alone.

The third series consisted of one experiment. This was made June 11 in Period XI after the patient had been several days on a diet of 19.7 gm. of protein, 94.1 gm. of fat, and 12.3 gm. of carbohydrate and after the urine had become sugar-free and the acquired sugar tolerance was approximately 30 gm. It is assumed there-

fore, that not only the protein quota of sugar but also all the ingested carbohydrate was being burned. The test meal consisted of 6.5 gm. of protein, 31.4 gm. of fat, and 4.1 gm. of carbohydrate, which is not far from two-thirds of the test meal of the first series.

The variation of successive tests during the forenoon without food is shown in the control experiment of the first series, May 27. The basal metabolic rates observed were -11, -15, -10, -8, and -12 per cent, an average of -11.2 per cent with an average variation of 1.8 and an extreme variation of 3.8 from the mean. The respiratory quotients were 0.67, 0.69, 0.68, 0.71, and 0.72, an average of 0.694 with an average variation of 0.018 and an extreme variation from the mean of 0.026. It is not unlikely that there was a slight underventilation of the lungs in the first three experiments with a corresponding compensation during the last two.

As may be seen in Table II there is a slight difference in the metabolic rate following the ingestion of food in each of the four experiments of the first series; however, it seems best to discuss the average results for these experiments. The basal rate before the test meal averaged -12 per cent; $\frac{1}{2}$ hour after the meal $+8$ per cent; 1 hour after, $+5$ per cent; 2 hours after, $+10$ per cent; and 3 hours after, $+6$ per cent. Therefore, in the first series of experiments, as a result of the specific dynamic action of 8.8 gm. of protein, 50.5 gm. of fat, and 14.2 gm. of carbohydrate, the metabolic rate was raised above the basal level 20, 17, 22, and 18 points $\frac{1}{2}$, 1, 2, and 3 hours, respectively, after ingestion; the rise was prompt and remained at essentially the same increased level for at least 3 hours.

The respiratory quotients of the postprandial observations showed no elevation whatsoever from those of the preprandial, completely failing to mirror the effect of the combustion of 14.2 gm. of carbohydrate. Indeed in the second and fourth experiments of the series the respiratory quotients are actually depressed immediately after the meal, rising again to the preprandial level only after the 3rd hour. The basal respiratory quotient (0.74) of the first experiment may be discarded for the reason that the patient was startled during the test by the dropping of a tray.

In the first two experiments of the second series the normal level of the basal metabolic rate averages -3 per cent. The test meal con-

sisted of 34.9 gm. of protein, 42.1 gm. of fat, and 1.3 gm. of carbohydrate. ½ hour after its ingestion the metabolic rate increased to an average of +11, after 1 hour to +11, after 2 hours to +22, and after 3 hours to +20 per cent. The specific dynamic action of the food, therefore, increased the metabolic level 14, 14, 25, and 23 points above the basal level ½, 1, 2, and 3 hours, respectively, after the food was taken. This increase was superimposed upon a base line which was 9 points higher than that of the first series of experiments. Comparison with the first series shows that the effect was less rapid but slightly higher with an apparent tendency to be more prolonged. It is possible that this difference in action may be due to the larger proportion of protein in the test meal.

The behavior of the respiratory quotients observed in the second series was surprising. No postprandial elevation was anticipated because the D:N ratio justified the assumption that glucose combustion during the period was minimal, but no such depression of the quotient as occurred was expected. In both experiments this is unmistakable; there was a drop from preprandial levels of 0.69 and 0.68 to 0.61 and 0.62, ½ hour after the meal, remaining as low as 0.64 and 0.66 after 3 hours. In the light of the marked reaction observed in these two experiments the slight postprandial depression of the quotients in two of the experiments of the first series assumes significance as does that observed in the single experiment of the third series.

The third experiment of the second series in which the test meal consisted of 25 gm. of glucose was conducted primarily as a control of the completeness of the loss of sugar tolerance. The D:N ratios of the previous 3 days had been close enough to the theoretic ratio of 3.65 to justify the assumption that the patient was a total diabetic. The effect of the sugar on the rate of metabolism was so slight that interpretation is difficult. An elevation of 5 and 8 points was noticeable ½ and 1 hour after ingestion, but this was not much greater than the fluctuations of the rates recorded May 27 when no food was given. The respiratory quotients remained the same or were slightly decreased after the ingestion of glucose and are, therefore, evidence against its immediate combustion. The D:N ratio for this day, 1.32, suggests, it is true, that some glucose was burned. This ratio, however, reflects the metabolism of the entire 24 hours and

a more probable explanation is that resumption of glucose oxida-
tion occurred at some time later in the day as the result of the
beneficial effect of the lower protein intake. It may also indicate
the retention of some of the ingested sugar to resupply depleted
glycogen stores.

The single experiment of the third series conducted with a test
meal of 6.5 gm. of protein, 31.4 gm. of fat, and 4.1 gm. of carbo-
hydrate revealed the same phenomena observed after the larger
test meals of the first and second series. The metabolism rose
14, 17, 11, and 9 points $\frac{1}{2}$, 1, 2, and 3 hours after ingestion of food,
the curve being less high and less prolonged than those of the two
former series. This smaller reaction is probably explained by the
smaller size of the test meal.

The respiratory quotients obtained in this experiment exhibit
the same behavior noted in two of the experiments of the first
series and unmistakably in the two experiments of the second
series, namely, a postprandial depression. This is slight and
would be insignificant were it not for the previous observation.

We will not attempt, at this time, the interpretation of these
postprandial depressions of respiratory quotients although we
believe they are significant because they occurred in five of six
good experiments, or six times in seven, if the doubtful basal
quotient in the first series is included. It may be noted, however,
that the depressions of the quotients are so much greater after
high protein meals as to suggest that they may represent a
protein effect.

The Basal Metabolic Rate.

During Period I of the observations the basal metabolic rate
averaged for eight determinations -12 per cent and the daily
variations were slight. The protein in the diet was 48.8 gm. and
the patient's glucose tolerance was limited as shown by an average
D:N ratio of 1.52.

In Period III the basal metabolic rate was 8 points lower than
in Period I. The reason for this lower rate is not quite clear.
Period III was preceded by a 2 day fast with resulting decrease
in the amount of glucose eliminated in the urine, a lower D:N
ratio, and increased sugar tolerance; the lower basal metabolic
rate seems primarily dependent on the general benefit obtained

by the fast, as indicated by the rate of -27 on April 21 (end of Period II); however, the fact that the main alteration in the diet of Period III was a decrease in the carbohydrate intake, must not be overlooked as a factor.

Although there is legitimate doubt as to the cause of the fall in the basal metabolic rate in Period III there is no question with regard to the interpretation of the marked and sudden rise from an average of -20 per cent of Period III to the average of -14 per cent found in Period IV. This rise definitely followed the increase of approximately 50 gm. of protein, making a total intake of 94.2 gm., 3.0 gm. for each kilo of body weight. Acidosis developed subsequently to the rise in the basal metabolic rate and did not materially exceed that of Period I so that acidosis did not seem to be the cause. The average for Period IV of -14 per cent was materially lowered by the observation made April 30 which is so far below the average for the period that the accuracy of the determination may be questioned.

The increase in the basal metabolic rate in Period V over that of Period IV is slight especially if the determination of April 30 of -19 per cent is excluded from the average of the latter. Therefore, the increase of approximately 40 gm. of fat, the protein and carbohydrate intake remaining practically the same, increased the basal metabolic level only 2 (or 4) points from -12 (or -14) to -10 per cent. The D:N ratio rose to an average of 3.73 in this period. Acidosis was simultaneously increased. From an examination of the data for this and the preceding period the conclusion seems warranted that the increase in the basal metabolic rate was not caused at least in major part, either by the fat in the diet or by the acidosis that developed later, but was mainly, if not entirely, caused by a cumulative specific dynamic action of protein (25, 26, a).

The low protein and low carbohydrate with relatively high fat, Newburgh type of diet of Period VI, allowed the basal metabolic rate to fall quite rapidly to -20 per cent (May 13) from the previous high level of -10 per cent. This diet contained approximately 10 gm. of protein, 83 gm. of fat, and 16 gm. of carbohydrate which totalled only 881 calories. The food ingested exceeded the basal requirement (856 calories), but since it did not allow a sufficient excess to balance the 10 per cent needed for the specific dynamic action of the food and 10 per cent for the movements in

bed, it was below the maintenance requirement at this level. Therefore, on this diet the patient had in all probability to draw on her own reserves for approximately 147 calories, or about 16 gm. of endogenous fat daily; however, as there was a negative nitrogen balance of 2 to 3 gm. half of this deficit was probably made up by the combustion of endogenous protein. The acidosis decreased, but apparently more slowly than the basal metabolic rate and does not, therefore, appear to have been a factor in producing the drop in metabolism observed. In the production of the decreased basal metabolic rate we have, then, two factors, the elimination of the cumulative specific dynamic action of a high protein diet, and the reduction of the calorie intake to a subnormal level of maintenance.

The second of these factors was eliminated in Period VII when the diet was increased to 30.7 gm. of protein, 109.1 gm. of fat, and 14.7 gm. of carbohydrate totalling 1,200 calories which was within 84 calories of the patient's normal daily requirement (normal basal requirement of 1,070 calories plus 10 per cent for specific dynamic action and 10 per cent for movements) and was 172 calories in excess of her needs calculated on a basal metabolic rate of -20 per cent. The average basal metabolic rate remained unaltered at -20 per cent despite this increase in the diet so that the sudden reduction in the basal metabolic rate in Period VI seems to be owing primarily to the elimination of the protein which had temporarily raised it above the low level which had become customary for this patient.

The diet of Period VIII was constructed to make it practically isocaloric with the high protein, high fat diet of Period V and totalled 1,692 calories. This diet was 408 calories in excess of the normal daily needs of this patient at rest which was 1,284 calories (1,070 plus 10 per cent for specific dynamic action and 10 per cent for muscular movement). As a result of this new diet the basal metabolic rate rose 6 points from an average of -20 to -14 per cent. It is difficult to determine the specific cause of this rise as there was an increase of 42.3 gm. in the amount of fat and also an increase of 27.9 gm. in the amount of carbohydrate in the diet. The carbohydrate as such, and that from protein, therefore, was 58.0 gm. [$42.6 + (26.5 \times 0.58) = 58.0$] which was about twice the patient's best sugar tolerance.

These two factors caused a moderate break in the sugar tolerance. The rise in the basal metabolic rate in this instance was obviously not owing to the protein, and might or might not have occurred from an increase in the fat if the carbohydrate intake had remained constant. The increase in acidosis was slight and did not precede the rise in the basal metabolic rate sufficiently for us to attribute the cause to the acidosis. While it is impossible, therefore, to draw conclusions with regard to the exact reason for the effect of this diet, it seems probable that its high calorie value was in part responsible. This is in agreement with the arguments of Allen against the Newburgh diets and these experiments support the validity of his objections when such diets exceed a certain optimal calorie value.

The fast of 2 days in Period IX caused a rapid improvement in the acidosis and a decrease in sugar elimination. The basal metabolic rate, however, did not fall as it did after the previous fast (Period II) nor was the improvement in the sugar tolerance sufficient materially to decrease the D:N ratio. Some factor or accumulation of several factors in the diet of Period VIII apparently produced not only an immediate elevating effect in the rate, but one from which the recovery was slow.

The diet of Period X was essentially the same as that of Period V, namely, high protein and high fat. The previous low protein, high fat diet of Period VIII may have produced a more susceptible status, as we have pointed out, but in any case the reaction to the diet of Period X was so marked and so rapid contrasted to that of Period VIII that no doubt can exist as to the harmfulness of excessive protein. The D:N ratio of total diabetes resulted almost at once, and paralleling this fall in sugar tolerance the basal metabolic level rose to an average of −4 per cent. This is 9 points above the level determined May 30, and from 12 to 16 points higher than that of the periods in which the patient seemed to be at the metabolic level best suited for her condition (Periods III, VI, VII, and XI).

In Period XI there was a big reduction in protein from 105 to 20 gm. and in fat from 126 to 94 gm. with an increase of carbohydrate to 12.3 gm. Therefore, the good effect of this diet must be credited in great part to the reduction in protein; however, if contrasted with the results produced by the diet of Period VIII

it is further evident that the lower fat and a total carbohydrate intake slightly less than her tolerance [12.3 + (19.7 × 0.58) = 23.7] also was beneficial.

Throughout these experiments the basal metabolic rates varied inversely with the general condition of the patient. During Periods III, VI, and VII when the rates were lowest the glucose utilization was best and acidosis was either controlled or decreasing. When the basal metabolic rates rose as in Periods IV, V, and X sugar tolerance diminished and acidosis increased. This parallelism between the degree of depression of the basal metabolic level and the rise of the D:N ratio is shown graphically in Chart 1.

Respiratory Metabolism of Thirty-One Patients with Diabetes.

In 1910 and 1912, Benedict and Joslin (6, 7, 8) published extensive studies of the basal metabolism in diabetes from which they concluded that the basal metabolic rate was increased in severe diabetes. Allen and Du Bois (3), however, have shown that the normals which were used by Benedict and Joslin averaged 8.6 per cent below the Du Bois normal standards and that the nineteen patients studied by Benedict and Joslin were only 2 per cent above the Du Bois normal, although 11 per cent above the normal controls selected. Allen and Du Bois also pointed out that of the twenty-six carefully investigated cases of diabetes reported up to that time, the basal metabolic rates were normal in thirteen, increased in nine, and decreased in four.

We have recently investigated the basal metabolism of thirty-one patients with diabetes. A brief summary of the data obtained is given in Table III. The basal rates were found to be within 10 per cent of the Du Bois normal standards in 10 out of 12 uncomplicated cases without emaciation; the other two had decreased rates. Body weights of 20 per cent or more below the standard weight in medico-actuarial mortality tables (28) are interpreted as indicating prolonged subnormal diets; the patients, with uncomplicated diabetes but with these low body weights, have, with only one exception, shown depressions of their basal metabolic rates of from −11 to −32 per cent, the degree of depression being possibly roughly proportional to the degree of loss of weight below the standard. Two emaciated patients, Cases A350440 and

TABLE III.

Respiratory Metabolism of Thirty-One Patients with Diabetes.

Case No.	Sex.	Age.	Diagnosis.	Known duration.	Date of test.	Basal calories each hour.	Basal metabolic rate.	Height.	Weight.			Average diet, previous 3 days.		N of the urine.
									Naked.	Stand-ard.*	Below stand-ard.	Protein.	Calo-ries.	
		yrs.			1920		per cent	cm.	kg.	kg.	per cent	gm.		gm.
A336236	F	29	Acute diabetes.	2 years.	Nov. 16	34.3	−28	157.6	34.9	54.0	35	50	732	
					" 17	31.6	−32		33.7					
A375561	F	31	Acute diabetes.	8 months.	1921 Nov. 8	43.5	−12	163.3	38.8	58.0	33	14	902	5.3
					" 9	42.7	−13		38.8					
A359373	M	57	Diabetes, arteriosclerosis.	4 years.	June 6	45.8	−17	168.0	44.2	67.5	35	60	1,586	
					" 13	42.9	−22		43.7			60	1,557	
					" 16	45.7	−17		43.8			60	1,545	
					" 18	42.8	−21		42.8			60	1,529	
A367133	M	23	Acute diabetes.	4 years.	Sept. 12	47.6	−17	169.5	42.3	62.0	32	10	902	4.4
					" 13	45.0	−21		41.8			10	902	4.4
					" 13	47.3	−17							
					" 14	46.6	−18		42.3			10	902	4.2
					" 14	45.7	−20							
A370281	M	14	Acute diabetes.	1 month.	Sept. 12	41.4	−27	157.4	33.1	47.0	30	10	902	4.1
					" 13	40.1	−29		33.1			10	902	
					" 13	39.9	−30							

A350440	M	38	Acute diabetes.	1 year.	Feb. 24	60.6	+ 3	168.0	45.9	65.0	29	100	3,000	
					" 25	59.6	0		46.6		.	100	3,000	
					Mar. 3	47.1	−22		47.7			0	0	
					" 3	46.1	−23					0	0	
					" 4	47.0	−21		47.2			0	0	
A374395	M	15	Acute diabetes.	8 months.	Oct. 13	50.9	−13	156.8	36.1	48.0	25	40	1,006	10.3
					" 14	51.1	−13		35.4			40	1,006	10.3
					" ..	45.2	−22		33.8			40	1,489	9.0
					" ..	48.6	−15		33.8			40	1,489	9.0
A341010	F	28	Acute diabetes.	8 months.	Apr. 28	46.9	− 9	163.1	41.3	57.0	28	10	983	
					Sept. 28	52.8	0		42.8			?	?	
A365349	F	16	Acute diabetes.	3 months.	Aug. 12	48.0	−16	168.8	41.5	57.0	27	30	1,313	4.4
					" 15	47.1	−17		41.3			30	1,313	
A355389	M	37	Diabetes, arteriosclerosis.	5 years.	Apr. 7	46.7	−24	168.0	49.5	65.0	24	50	349	
A352014	M	42	Acute diabetes.	1 year, 2 months.	Mar. 10	65.2	+15	162.5	47.4	62.0	24	100	3,000	
					" 11	58.7	+ 4		47.3			100	3,000	
					" 17	53.5	− 9		50.2			0	0	
					" 18	53.4	− 8		50.0			0	20	
					" 30	46.8	−17		46.1			70	1,144	
					" 31	46.5	−18		46.8			70	1,144	

* From Medico-actuarial tables (28). Tables V and IX were used allowing 1 inch in height for shoes, and 10 pounds for the weight of clothes in men and 7 pounds in women.

TABLE III—Continued.

Case No.	Sex	Age	Diagnosis	Known duration.	Date of test.	Basal calories each hour.	Basal metabolic rate.	Height.	Weight.			Average diet, previous 3 days.		N of the urine.
									Naked.	Stand-ard.*	Below stand-ard.	Protein.	Calo-ries.	
		yrs.			1921		per cent	cm.	kg.	kg.	per cent	gm.		gm.
A353464	M	33	Acute diabetes.	3 months.	Apr. 11	52.1	−19	175.0	54.0	70.0	23	54	905	
A353448†	M	17	Acute diabetes.	1 year, 8 months.	Sept. 1	52.1	−26	179.4	51.8	61.5	20	70	1,764	11.6
A366769	M	13	Acute diabetes.	11 months.	Sept. 1	39.0	−24	145.8	24.3	?		10	902	
A344032	F	9	Acute diabetes.	10 months.	Sept. 5 " 6	45.6 41.5	−16 −24	137.8	26.0 25.6	? ?		15 15	1,506 1,506	4.3 2.8
A363938	F	51	Diabetes, ex-ophthalmic goiter.	3 years.	Aug. 4	64.5	+39	153.0	41.3	58.0	29	45	1,738	
A354364‡	F	21	Diabetes, ex-ophthalmic goiter.	1 month.	Apr. 15 " 26 May 18 " 19	67.7 72.8 81.1 65.3	+34 +45 +47 +27	157.5	41.4 41.9 43.4 43.3	53.0	22	60 60 ? ?	1,829 1,829 ? ?	
A375292	F	61	Diabetes, ade-noma of the thyroid (hy-perthyroid-ism?).	1 year, 7 months.	Nov. 3 " 5 " 5	64.3 61.0 59.9	+17 +11 + 9	162.5	60.0 59.9	61.0	6	14 14	902 902	5.9 6.2

No.	Sex	Age	Diagnosis	Duration	Date								
A367201	F	53	Diabetes, adenoma of the thyroid.	1 month.	Aug. 10	66.3	+6	158.5	76.5	61.0		60	1,419
					" 11	68.7	+10		76.8			60	1,419
					" 26	66.6	+8		74.6			60	1,419
					Sept. 5	65.1	+6		73.0			60	1,419
A352452	F	47	Diabetes, arteriosclerosis.	1 year.	Mar. 22	48.8	−16	159.0	61.1	60 0		60	1,144
					" 24	53.8	−7		60.0			60	1,214
					" 30	47.5	−17		59.0			80	1,277
A374229	M	63	Diabetes, arteriosclerosis.	Unknown.	Nov. 14	67.7	+3	168.5	72.0	67.5		54	1,923
					" 15	55.4	−16						
A351994	F	51	Diabetes, arteriosclerosis.	1 year, 6 months.	Mar. 15	53.5	−1	153.0	58.4	58 0		46	1,199
					May 16	49.0	−7		55.5			70	1,288
A353379	F	58	Diabetes, obesity.	5 months.	Apr. 27	59.0	−3	155.7	75.6	60.0		20	1,470
A352965	F	62	Diabetes, fibroma of the uterus.	1 month.	Mar. 30	51.0	−1	154.5	55.0	60.0	8	20	1,240
A268177	M	47	Diabetes innocens.	4 years.	Apr. 25	74.7	−1	172.1	83.0	71.0		60	1,800

† Diet same, previous 6 months.
‡ Thyroidectomy May 31, 1921; hypertrophic parenchymatous thyroid, weighing 20 gm.

TABLE III—*Concluded.*

Case No.	Sex	Age	Diagnosis.	Known duration.	Date of test.	Basal calories each hour.	Basal metabolic rate.	Height.	Weight. Naked.	Weight. Standard.*	Weight. Below standard.	Average diet, previous 3 days. Protein.	Average diet, previous 3 days. Calories.	N of the urine.
		yrs.			1921		per cent	cm	kg.	kg.	per cent	gm.		gm.
A373176	M	53	Diabetes, obesity, arteriosclerosis.	8 months.	Oct. 13	86.2	+ 5	168.0	111.9	67.0		60	1,419	7.0
					" 14	84.1	+ 3		111.5			60	1,419	
					" 27	86.1	+ 6		109.9			60	1,400	
					" 28	78.5	− 3		110.3			60	1,400	
A350936	M	42	Diabetes, obesity.	Unknown.	Mar. 7	86.4	+ 3	178.8	100.1	75.0		60	1,810	
A376588	F	48	Diabetes, arteriosclerosis.	Unknown.	Nov. 16	75.2	+ 6	169.5	87.5	69.5		60	1,755	8.9
					" 17	75.2	+ 6		87.6			60	1,755	8.7
A356478	F	36	Diabetes, obesity.	Unknown.	May 6	76.6	+ 7	159.1	95.9	56.0		60	1,349	
A355796	M	40	Diabetes innocens.	3 years.	Apr. 25	77.1	+ 8	173.8	72.3	69.5		60	1,700	
A356694	F	33	Diabetes, obesity.	Unknown.	May 4	89.5	+ 6	175.7	116.2	68.0		60	2,000	

A352014, had rates of +3 and +15 per cent, respectively, on the 2 or 3 days following their admission to the hospital. Both of them had been eating a large amount of food including liberal quantities of protein. In each case the rate was promptly depressed by the institution of low diets. The well nourished and the obese patients, although many of them had fallen in weight below their previous maximal weights, did not show any depression of their basal metabolic rates despite dietary deficiencies. Presumably obese persons maintain a normal metabolic level on low diets at the expense of calories derived from endogenous sources. The only elevated rates occurring in the series are those attributable to complicating hyperthyroidism (Cases A363938, A354364, and A375292).

We also reviewed the available observations of others and can find in them but few deviations of the basal metabolic rates of patients with uncomplicated diabetes from the Du Bois normal standards, that may not be attributable to factors which would be equally effective in modifying the metabolic rates of non-diabetic persons. This is in agreement with observations of Bernstein and Falta (10). The majority of patients with severe diabetes are emaciated and on a low calorie food supply. If other factors are negligible they will reveal basal metabolic rates 15 to 30 per cent below the Du Bois normal for persons of like surface area, age, and sex. The same is true for non-diabetic persons, as shown by the observations of Zuntz and Loewy (22, 48) on themselves and those of Benedict, Miles, Roth, and Smith (9), on squads of Springfield students. The emaciated diabetic patient with a low basal metabolic level will react, however, to a high protein dietary with an increase in this level, the result apparently of a cumulative effect of the specific dynamic action of the protein. To a less extent, a similar reaction is obtained by a high calorie, low protein diet. The same phenomenon as the first of these at least, namely the cumulative specific dynamic action of protein, is observed in normal dogs and men (25). This would seem to account for the normal or above normal basal metabolic rates obtained in emaciated diabetic patients who are studied during periods of high protein diets. Finally, as Woodyatt (45) suggests, when a diabetic patient is undernourished to the point of extreme cachexia his endogenous metabolism consists largely of

protein, his fat stores being depleted. Such a patient on a diet low in calories actually metabolizes daily a large amount of protein even though he is receiving very little protein in his food and his rate will rise accordingly to a level that would be normal or even above the normal for a patient of like surface area, age, and sex. This phenomenon is exactly that observed by Loewy on himself during the year 1917. As a result of food restriction(26, *a*) Loewy lost gradually in weight during 1916 and the early part of 1917 and simultaneously his heat production fell, but in July, 1917, when he had lost 22 per cent of his former weight and when the total calories and protein of his diet were low, his heat production suddenly rose again to a level above that observed in 1914. On the day of the observation the nitrogen of the food was 8 gm. and that of the urine 17 gm. Zuntz and Loewy (48) compared this result with precisely similar observations made by Zuntz (47) on an undernourished, but otherwise normal dog.

At least one factor, however, enters into the problem in the case of the diabetic patient, namely acidosis, which is not present or at least not present to the same degree in the non-diabetic person. This may tend to elevate the metabolism of the diabetic patient although the evidence (7) for this is inconclusive, as was shown by Allen and Du Bois (3), and as the experiments here reported reveal. Bernstein and Falta (10) concluded from their study of diabetic patients 'with acidosis that acidosis has very little if any effect on the general metabolic level.

If it is true that the basal metabolism of the uncomplicated diabetic person is normal as judged by the Du Bois standard, except when it is reduced by dietary, particularly protein, restriction and if it is desirable therapeutically to maintain this level below normal in the severer cases, as the experiments on Bessie B. seem to indicate, it follows that the observation of a basal rate equal to or higher than the Du Bois normal in an emaciated patient should be taken as a warning that the available food of the diet is excessively rich in protein (or calories) or so low in calories that the patient is in a critical state of starvation and calling on his body protein to meet energy requirements.

The Dextrose-Nitrogen Ratio.

We are aware that many persons will not accept the validity of D:N ratios obtained with diets containing carbohydrate. The objection does not seem valid under such circumstances as existed in these experiments in which daily observations are conducted over long periods and the carbohydrate intake is accurately known. However, in order to eliminate objections on this score as much as possible, the diets of Periods III, IV, V, and X were constructed with insignificant amounts of carbohydrate.

The ratio 3.65 which was maintained by Bessie B. 4 days on two occasions (Periods V and X) indicates the non-utilization and excretion of all the sugar theoretically derivable from the protein metabolism. Lusk (23) has contended that this is the maximal ratio obtainable and that the absence of higher ratios in satisfactory experiments is evidence against the assumption of the derivation of sugar from fat. It would have been desirable to continue Periods V and X for 3 or 4 days longer, but this appeared to be unjustifiable. The conditions preceding each of these periods were such that the previous storage of sugar is unlikely and the data from urinalysis obtained on the days which immediately followed give no evidence of any failure in nitrogen excretion. The deviations of the ratios actually observed in Periods V and X from Lusk's figure, 3.65, are within the limits of error of physiologic experiments.

Mandel and Lusk (27), in 1904, presented the first case of diabetes in man observed with a D:N ratio of 3.65 and referred to this ratio as the critical or fatal ratio, maintaining that it indicated such extreme severity of the diabetic process that much improvement of glucose tolerance or material prolongation of life was precluded. In more recent years, Lusk (20, 23) has felt less certain of the prognostic significance of the critical ratio. A case described by Allard (1, 23) showed improvement on fast days and the carefully studied patient, Cyril K., of Geyelin and Du Bois (21), discussed in detail by Allen and Du Bois (3) recovered a carbohydrate tolerance of 250 gm. The diabetes in this case, however, was complicated by infection. The leucocyte count at the time of the critical ratio was 15,500. Furunculosis existed, an abscess was incised, and pus was evacuated. Recovery of sugar tolerance was coincident with control of this infection. From the descrip-

tion, the case was of the acute type, intrinsically severe in that the ultimate prognosis in this type of diabetes (41) is bad, but in an early stage (2 months) when carbohydrate tolerance usually remains high unless temporarily depressed by complications. It is, therefore, not surprising that Cyril K. regained a high tolerance after incision of abscess and control of infection. We are of the opinion that the significance of the critical ratio in such a case is primarily that of the complication and less that of the underlying diabetes, whereas, in uncomplicated diabetes it has more nearly the prognostic importance which Lusk originally attributed to it.

The Specific Effect of Protein on the D:N Ratio and on the Rate of Sugar Utilization.

It is an old and well established clinical observation that diabetic patients do poorly on an excessive protein intake, and recently, Allen and Du Bois (3) secured on at least two occasions the D:N ratio of 3.65 indicating complete diabetes as a result of a diet of 119 gm. of protein, 41 gm. of fat, and 23.5 gm. of carbohydrate. There are several explanations for this fact, none of which are entirely satisfactory. One is that diets rich in protein are harmful, like all luxus diets, because of their high food value. Another is that a large portion of the protein molecule is convertible into sugar and that feeding high protein is, therefore, equivalent to taxing the sugar tolerance with large amounts of sugar. A third is based on the probability that certain of the amino-acids notably leucine, phenylalanine, and tyrosine are converted into acetoacetic acid, so that, feeding protein favors ketogenesis and thereby indirectly lowers sugar tolerance.

The experiments under consideration were stimulated by the recent researches of Professor Graham Lusk and Dr. Eugene F. Du Bois. They were planned primarily to investigate this question. In Period V and again in Period X as we have mentioned, feeding high protein diets to Bessie B. resulted in complete suppression of sugar utilization, the urine for these periods exhibiting the D:N ratio of total diabetes. The high energy values of these diets do not alone explain the high ratios. The low protein, high fat diet of Period VIII was practically isocaloric with the high protein diets in Periods V and X, yet the high fat diet had relatively little effect on sugar tolerance when compared to that

of the high protein diets. Nor does it appear that the sugar derivable from the protein is alone responsible inasmuch as the high fat diet of Period VIII contained carbohydrate equal in glucose value to the sugar derivable from the protein in the two high protein diets. Likewise, the ketogenic amino-acids of the protein do not account for the difference between the periods. In Period IV the D:N ratio reached 2.98 before acidosis was appreciable and in Period X sugar tolerance was lost completely before ketogenesis was much accelerated. Furthermore, the high protein diet of Period X was so planned that its ketogenic value did not materially exceed the ketogenic value of the fat diet of Period VIII and yet its effect on sugar tolerance was definitely greater than that of Period VIII.

It would seem, therefore, that protein exerts some specific depressant action on the sugar-utilizing mechanism of the diabetic patient, in addition to such effect as may be due to the sugar and ketogenic substances, which the ingestion of protein throws upon the metabolism. This effect of protein is appreciably greater than the effect produced by the ingestion of an isocaloric amount of fat and carbohydrate. It may be related to the cumulative specific dynamic action of protein on the basal metabolism. The basal metabolic rates in these experiments varied inversely with the sugar tolerance and in Periods V and X when, as a result of high protein feeding the basal metabolic level had been elevated most, the sugar utilization was depressed to zero.

Ketogenic Antiketogenic Ratios.

The rapid reduction and control of ketogenesis by the diets of Periods VI and XI is of interest in connection with recent discussions of ratios between the acetone formers and the glucose value of non-ketogenic food mixtures.

Included among the acetone formers are the fatty acids and certain amino-acids, particularly phenylalanine, leucine, and tyrosine. The glucose value of a diet according to Shaffer (33, 34) and Woodyatt (45) is the sum of all the carbohydrate, 58 per cent of the protein, and about 10 per cent of the fat. Shaffer calculates the probable number of molecules of each of these two groups of substances and, by dividing the former by the latter, obtains a ratio which he refers to as the ketogenic antiketogenic

ratio. From chemical experiments and clinical studies Shaffer concluded that this ratio must not exceed 1:1 if incomplete combustion of fat and excretion of acetone bodies are to be avoided. Woodyatt (43), on the basis of theoretic considerations had previously reached similar conclusions.

May 12, in Period VI the 24 hour acetone excretions of Bessie B. (total acetone bodies expressed as acetone) had fallen to 2.39 gm. The ammonia nitrogen excretion was 0.54 gm. and the acetone of the blood 19 mg. by May 10. These figures are not minimal, but at the time they were daily becoming smaller. The carbon dioxide-combining power of the plasma was normal. The total energy exchange for this day may be calculated with fair accuracy from our knowledge of the basal metabolism taken before breakfast the following morning. This was 36 calories for each hour or 864 calories for a 24 hour period. Adding 10 per cent for specific dynamic action of the food and 10 per cent for movements in bed a total is obtained of 1,037 calories. Assuming, in view of the previous low carbohydrate diet, that all of the ingested carbohydrate (15.6 gm.) was burned and that the fat burned is represented by the difference between the total calories and the sum of the calories from protein and carbohydrate, we may calculate the ketogenic antiketogenic ratio of this day's metabolism according to Shaffer (34).

<div align="right">calories</div>

Calculated from nitrogen of the urine (4.84×26.5)....... 128.26
From carbohydrate (15.6×4.1)......................... 63.96

     Total from protein and carbohydrate................. 192.22
$1,037 - 192 = 845$ calories from fat.
$\dfrac{845}{9.3} = 90.9$ gm. fat.

	Gm. molecules of ketogenic substance (fatty acids, etc.).	Gm. molecules of antiketogenic substance (glucose).	Ratio.
Carbohydrate...		0.0867 (15.6×0.00556)	
N of the urine...	0.0484 (4.84×0.01)	0.0968 (4.84×0.02)	
Fat.............	0.3118 (90.9×0.00343)	0.0518 (90.9×0.00057)	
Total.........	0.3602	0.2353	1.53:1

The ratio 1.53:1 is abnormally high for Shaffer's assumption that a 1:1 ratio should exist if ketogenesis is to be avoided; each gm.

molecule of ketogenic substance such as fatty acid, requires, according to his assumption, a gm. molecule of antiketogenic glucose.

Again, following Period X, a dangerous and rising acidosis was checked by a low protein, high fat diet. On this occasion the diet more nearly met the maintenance requirements of the patient with regard to protein and calories, than was the case May 12. June 11, carbon dioxide-combining power of the plasma was 46.6 volumes per cent; 3 days later it had reached 54 volumes per cent. The total acetone value of the blood of June 11 was 24 mg. for each 100 cc. and the total acetone bodies excreted in the 24 hour specimen of urine of June 10 had fallen to 2.95 gm. from much higher values. The ammonia nitrogen excretion was 0.80 gm. The urine contained between 6 and 7 gm. of sugar which is disregarded in the following calculations because the urine of the next day was sugar-free and it is possible that the few grams excreted June 10 represented merely delayed excretion of previous accumulation. If this sugar were not disregarded the result of the following calculation would be still less favorable to Shaffer's assumption of a 1 : 1 ratio. The qualitative tests for diacetic acid and acetone in the urine were negative. The metabolism is known for June 10 from the respiratory data obtained on the following morning and the ketogenic antiketogenic ratio may be calculated in the same manner as in the previous example. It is assumed that all of the carbohydrate ingested, 12.3 gm., was burned.

	calories
Basal metabolism for 24 hours June 11, 1921 (37.9 × 24)..	909.6
Specific dynamic action of food (909.6 × 0.10)...........	90.96
Requirement for movements in bed (909.6 × 0.10)......	90.96
Total energy exchange...........................	1,091.52
Calculated from nitrogen of the urine (4.60 × 26.5).....	121.9
From carbohydrate (12.3 × 4.1)........................	50.43
Total from protein and carbohydrate..............	172.33

$1,091.52 - 172.33 = 919.2$ calories from fat.

$$\frac{919.2}{9.3} = 98.8 \text{ gm. fat.}$$

	Gm. molecules of ketogenic substance (fatty acids, etc.).	Gm. molecules of antiketogenic substance (glucose).	Ratio.
Carbohydrate...		0.0684 (12.3 × 0.00556)	
N of the urine...	0.0460 (4.6 × 0.01)	0.092 (4.6 × 0.02)	
Fat.............	0.339 (98.8 × 0.00343)	0.0564 (98.8 × 0.00057)	
Total.........	0.385	0.2168	1.78:1

The ratio observed in this instance is almost double that required for Shaffer's assumption of reaction between 1 molecule of ketogenic acid and 1 molecule of glucose; had a larger allowance been made for increased metabolism from movements, the calculation would have shown a ratio as high as 2:1. Since the patient spent about 6 hours of this day sitting in her chair and moving around her room, it is not unlikely that a slightly larger energy exchange than that calculated for the day did actually occur.

Woodyatt's (45) formulas for computing the maximal amount of fat, which can be included in a diet containing a limited number of grams of carbohydrate and protein without provoking ketosis, are based on the same assumption that Shaffer makes; namely, that each molecule of ketogenic acid will require a molecule of glucose to accomplish its complete oxidation. Lusk (26, a), on the other hand, recalculating Zeller's (46) data, hypothesized that 1 molecule of fatty acid might react with ½ molecule of glucose, that is, with a triose molecule. Ringer reached similar conclusions. Palmer's (30) data, obtained by studies on diabetic patients, would seem to substantiate this latter hypothesis. According to Lusk's hypothesis, Shaffer's ketogenic antiketogenic ratio would become 1:0.5, or 2:1, instead of 1:1.[1] The data obtained with Bessie B. and with other persons, both diabetic and non-diabetic, incline us to regard the 2:1 ratio as more nearly correct, at least under certain conditions. The degree of acidosis provoked when the ratio of ketogenic acid molecules to glucose molecules in the metabolism is not greater than 1:0.5 or 2:1 is at most very slight and insufficient

[1] In a recent paper presented before the Society of Biological Chemists Shaffer announced the conclusion that a 2:1 ratio in the actual combustion mixture might be accompanied by little or no formation of acetone bodies, but that when the proportion of acetoacetic acid formers to glucose was in excess of 2:1 excretion of acetone bodies occurred in an amount proportional to the excess over this ratio of ketogenic molecules.

to effect the metabolism adversely.   This has been true, however, only when the protein of the diet has been maintained close to 0.66 gm. for each kilo of body weight and when the total calorific value of the food has not exceeded the maintenance requirements of the patient.   A few exceptions have been noted; but thus far such exceptional cases have been complicated either by some infection or by a hyperfunctioning thyroid.   Woodyatt (44) has discussed the occurrence of occasional anomalous reactions encountered in diabetic persons which are difficult to explain in terms of the proportions of glucose and fatty acids undergoing simultaneous oxidation in the body.   He has pointed out that factors other than this play a part in ketogenesis, citing as examples certain types of infection which produce acidosis even in non-diabetic persons on balanced diets.   Our experience suggests that the proportion of fatty acid which will completely burn with a limited amount of metabolizing glucose is not the same at all metabolic levels, but may be higher when the level is depressed, as was the case with Bessie B.

CONCLUSIONS.

1. The complete metabolism of a patient with severe diabetes is reported for a period of 74 days.

2. The basal respiratory quotients averaged 0.693, the highest quotient being 0.74, and the lowest 0.65.   The significance of such quotients is discussed.

3. Nine experiments are reported on the immediate effect of food on the metabolic rate and respiratory quotient.   An unexplained depression of the quotients was observed to follow the ingestion of food, being most marked after large amounts of protein.

4. The postabsorptive or basal metabolic level of the diabetic patient is materially affected by the previous diet.   In the undernourished patient it may be found as low as 32 per cent below the Du Bois normal standards.   The ingestion of food containing 1 gm. of protein for each kilo of body weight with fat and carbohydrate in such an amount that the daily maintenance energy requirements of the patient were exceeded, caused an elevation of the basal level.   The ingestion of 3 gm. of protein for each kilo of body weight each day caused a greater rise in the basal metabolic

rate than occurred with isocaloric amounts of other foods. A cumulation of the specific dynamic action of protein seemed to account in the main for the elevation of the basal metabolic level when such occurred.

5. In diabetes, occurring in well nourished patients, the basal metabolic rate does not exceed the normal limits of Du Bois' standards, unless the patient is on a high protein (or high caloric) diet or has some complicating condition such as an infection or hyperthyroidism (adenoma of the thyroid or exophthalmic goiter). Acidosis itself does not appear to elevate the basal metabolic rate; an increase in the rate accompanied by an acidosis is probably due to the factor that causes the acidosis.

The basal metabolic rate is below the normal limits of Du Bois' standards in patients who are distinctly under weight and who have not been receiving a diet high in protein (or calories). The observations of a basal metabolic rate equal to or higher than the Du Bois normal in an emaciated patient should be taken as a warning that the available food of the diet is excessively rich in protein (or calories) or that the patient has been for so long on a diet so low in calories that as a result he is in a critical state of starvation and calling on his body protein to meet energy requirements.

6. The critical or fatal D:N ratio of 3.65 has the prognostic significance in diabetes originally attributed to it by Lusk, provided the diabetes is uncomplicated.

7. The sugar tolerance of the diabetic patient is depressed by high calorie, luxus diets, but much more markedly depressed by protein than by isocaloric amounts of fat. This protein effect is not primarily due to the sugar and ketogenic substances, which the ingestion of protein throws on the metabolism, but to some other more specific action of protein the result of which is to interfere with the mechanism of sugar utilization.

8. Throughout our experiments the glucose tolerance varied inversely with the basal metabolic level, rising as the rate fell and *vice versa*. This behavior seems to indicate a definite interrelationship of the two.

9. Diets high in fat but low in protein and planned so that they contained nearly 2 gm. molecules of fatty acid to 1 of glucose, on two occasions, checked a dangerous and rising acidosis. The

metabolism data of 2 days when the acidosis was minimal indicated actual ketogenic antiketogenic ratios in the metabolism mixtures of 1.53 and 1.78. It is suggested that the proportion of fatty acid which will completely burn with a limited amount of metabolizing glucose is not the same at all metabolic levels but may be increased by measures designed to depress the basal metabolic rate.

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THE INTERACTIONS OF OXYGEN, ACID, AND CO_2 IN BLOOD.

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In a recent paper (1) I have shown that the effect of acid or CO_2 upon the oxygen absorption curve of blood can be deduced quantitatively from the hypothesis that hemoglobin is a weak acid, dissociated electrolytically to a very slight degree, according to the equation,

$$H (Hb)_n \; \rightleftharpoons \; H^{\cdot} + (Hb)'_n$$
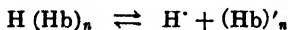
while oxyhemoglobin is a stronger acid, dissociated to a greater degree, according to the equation,

$$H (HbO_2)_n \; \rightleftharpoons \; H^{\cdot} + (HbO_2)'_n$$
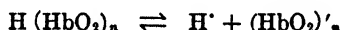
This hypothesis leads to a relation between the K of "Hill's equation" and the ratio of the hydrogen ion concentration (C_H) to the basic ion concentration (C_B) inside the corpuscle

$$\frac{1}{K} = \frac{1}{k}\left(1 + a\,\frac{C_H}{C_B}\right)$$

where k and a are constants. It can be shown that $a\,\dfrac{C_H}{C_B}$ is much greater than unity, so that the equation can be written, without sensible error,

$$\frac{1}{K} = \frac{a}{k}\cdot\frac{C_H}{C_B}$$

Taking logarithms, $\log K = $ pH $+$ constant, so that the relation between $\log K$ and pH is a straight line inclined at 45° to the axes. The linearity of this relation has been established experimentally by Barcroft (2) and by Donegan and Parsons (3),

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and it is noticeable that their curves are all inclined at approximately 45° to the axes; small divergences, however, do occur, and these are probably to be explained as follows. In the calculation of K for comparison with pH it has been assumed that $n = 2.5$. In any particular case this assumption may not be exact. In determining K the practice adopted by Barcroft has been to make an observation of the percentage saturation, at any oxygen pressure adjusted to give about half saturation. Assuming $y = 50$ the equation $\frac{y}{100-y} = Kx^n$ becomes $1 = Kx^n$, from which $\log x = -\frac{1}{n} \log K$. Thus if an incorrect value of n (say n') be assumed, an incorrect value of K (say K') will be calculated from it, the relation between the assumed and the true values being,

$$\frac{1}{n} \log K = \frac{1}{n'} \log K'$$

Thus the true value of K will be given by the equation

$$\log K = \frac{n}{n'} \log K' = \frac{n}{2.5} \log K'$$

In the cases, therefore, in which the relation between $\log K$ and pH is not inclined exactly at 45° to the axes it is obvious that the inclination can be adjusted to 45° by a suitable small alteration in the assumed value of n. There is further justification for the hypothesis. Many investigations have recently been made of the relation between the pH of blood and its CO_2 tension; if this relation be expressed in terms of C_H, instead of pH, it will be found that practically all the published curves become straight lines; the relation between C_H and pCO_2 therefore is linear, or nearly linear. Thus $\frac{1}{K}$ being a linear function of C_H must also be a linear function of pCO_2. Thus theory confirms the empirical relations established independently by Henderson (4) and Adair (5).

It should be noticed that the assumption as to the acid nature of hemoglobin differs from that commonly made, which is[1] that

[1] See Henderson (4), p. 408.

the unit[2] of hemoglobin containing 1 atom of iron is an acid HHb, the degree of electrolytic dissociation of which changes when it combines with oxygen. The assumption made here is that the molecule $(Hb)_n$ occurring in the formula which leads to Hill's equation of the dissociation curve, *viz.*,

$$(Hb)_n + nO_2 \rightleftharpoons (HbO_2)_n$$
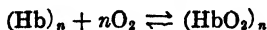
behaves as a mono-basic acid, dissociating according to the equation,

$$H (Hb)_n \rightleftharpoons H^\cdot + (Hb)'_n$$
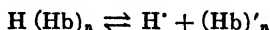
In this respect the dissociation of $H (Hb)_n$ is analogous to that of H_2CO_3, the first dissociable hydrogen ion alone of the complex molecule is involved in the dissociation, at any rate over the range of C_H possible in blood. Thus we do not necessarily assume that hemoglobin is not $(H Hb)_n$, but that over the range considered only the first hydrogen of the poly-basic acid is dissociable. It is possible that we should regard the acid hemoglobin rather as the acid sodium (or potassium) salt $H Na_{n-1} Hb_n$, but until we know more of the nature of the actions of bases on the hemoglobin molecule it would be idle to speculate further. In any case the assumptions (1) of the mono-basic properties of $(Hb)_n$, and (2) of the change wrought in the degree of electrolytic dissociation by the combination with O_2, are sufficient to account quantitatively for the effects of acids and CO_2 on the dissociation curves of blood.

We c-me now to the effects of oxygenation of blood upon its absorption curve for CO_2. The CO_2 absorption curves of oxygenated and reduced blood are known to be different (Christiansen, Douglas, and Haldane, 6) and this fact has naturally been credited to the greater electrolytic dissociation of oxyhemoglobin. Henderson (4) has attempted quantitatively to connect the effect of O_2 on the CO_2 absorption curve, with the effect of CO_2 (and acid) on the O_2 absorption curve, as he admits, however, only with partial success. There can be no doubt that, thermodynamically, the two sets of facts must be related, and should be deducible the one from the other. The difficulty in

[2] It is not justifiable to call this unit a molecule, unless it be shown that the unit $(Hb)_n$, which appears to act as a molecule, is a fiction.

my opinion has arisen from the assumption that hemoglobin in blood is ionized according to the formula H Hb $\rightleftharpoons$ H· + Hb′, and not H $(Hb)_n \rightleftharpoons$ H· + $(Hb)'_n$. With the latter assumption the quantitative connection between the two sets of phenomena is at once clear. At any considerable CO_2 pressure reduced hemoglobin in blood, being an exceedingly weak acid, is present as undissociated $H(Hb)_n$, while oxyhemoglobin, being a much stronger acid, is present as the salt B $(Hb\ O_2)_n$, holding onto the base B very firmly and denying it to the CO_2. Thus in combining with n molecules of O_2 the hemoglobin has seized on 1 molecule of base, and has deprived the blood, therefore, of the ability to combine with 1 molecule of CO_2. As Henderson very rightly points out[3] it is necessary to consider the "base transferred from carbonic acid to haemoglobin when fully reduced blood is fully saturated with oxygen *isohydrically*," *i.e.* at a constant hydrogen ion concentration, and he gives a figure from which it is seen that, in the blood of J. S. H., as the C_H increases the extra CO_2 taken up by reduced blood reaches a maximum of about 8.8 volumes per cent. Parsons (7) has investigated this subject by direct experiment, and from Fig. 4 of his paper,[4] it is seen that as the C_H increases the extra CO_2 taken up by his reduced blood, in excess of that taken up by his oxygenated blood at the same C_H, reaches a steady value of about 9 volumes per cent, thus entirely confirming Henderson's calculations with the blood of J. S. H. Assuming that the blood of J. S. H. had the normal O_2 capacity of 18.5 volumes per cent, we see then that the taking up of 18.5 volumes of O_2 has deprived the blood of base sufficient to take up 8.8 volumes of CO_2. Thus n should be equal to $\dfrac{18.5}{8.8}$; *i.e.*, to 2.1. Recent experiments by Brown and myself in this laboratory make it probable that the usually accepted value of n, *viz.* 2.5, is rather too high; that at any rate in the blood of sheep, oxen, or pigs, a value of n between 2.2 and 2.3 more exactly fits the facts. The agreement between this revised value and that calculated above from the $O_2 : CO_2$ ratio is very good, and appears a striking confirmation of the general validity of the hypothesis used to explain

[3] Henderson (4), p. 407.
[4] Parsons (7), p. 452.

the actions of acid and CO_2 upon the oxygen absorption curves of blood.

We see, therefore, that the hypothesis leading to the equation $y = \dfrac{Kx^n}{(1 + Kx^n)}$ bears the strain imposed upon it in explaining the effects of acid and CO_2 upon the O_2 absorption curve of blood, and in correlating these with the effects of oxygen upon the absorption of CO_2. It is clearly more than a mere mathematical chance that the equation is of such general application. It was deduced originally (8) from the hypothesis that salts affected the degree of "aggregation" of the colloid hemoglobin molecule. A precise theory, however, is not necessary; any factor which causes the hemoglobin in blood to exert an osmotic pressure equal to $\dfrac{1}{n}$ of the pressure of the oxygen with which it combines must necessarily lead to an equation of this type. Experiments, hitherto unpublished, made by Miss Atkinson in this laboratory, have shown that the osmotic pressure of hemoglobin in blood is about $\dfrac{1}{2.2}$ of the pressure of the O_2 combined with it. Thus far, therefore, the equation is necessary from the standpoint of thermodynamics; it remains to determine the actual chemical mechanism by which the osmotic pressure is lowered by the presence of salts. This mechanism is not yet clear and one can scarcely assent to an "aggregation" theory in its crude form; it is difficult to believe that a mere physical clumping together of the simple hemoglobin molecules, caused by a surface effect of salt on colloid, could have the extensive and exact results observed. I am inclined to the view that the electrolytic dissociation discussed in this paper is connected with these "osmotic" changes. If the real hemoglobin molecule be a complex weak poly-basic acid (like boric or phosphoric acid) its electrolytic dissociation, and the size of its molecule, may be largely influenced (according to the laws of mass action) by the presence even of small concentrations of basic or of hydrogen ions. My friend Mr. W. E. L. Brown prepared the attached note on the analogous case of boric acid, in which he shows that hydrolysis may largely affect the molecular weight of the boron-containing com-

plex, may in fact cause a chemical "aggregation" or "polymerization" of its molecules. In pursuing the subject, therefore, of the electrolytic dissociations of hemoglobin we are probably taking the shortest road towards a knowledge of the causes of those variations of osmotic pressure, which can be shown experimentally to exist, and whose existence necessarily involves the observed changes in the oxygen absorption curves.

The Effects of Hydrolysis on the Osmotic Pressure of Salts of Weak Acids.

By W. E. L. Brown.

If hemoglobin exists in the blood as the sodium or potassium salt of a weak acid it is reasonable to suppose that it must undergo a certain amount of hydrolysis, as do the salts of inorganic weak acids with strong bases; e.g., borates, cyanides, carbonates, etc.

It is stated by Mellor (9) that in a concentrated solution borax, $Na_2B_4O_7$, is hydrolyzed as follows:

$$Na_2B_4O_7 + 3H_2O \rightleftharpoons 2NaBO_2 + 2H_3BO_3$$
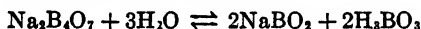
It is also stated that sodium metaborate, $NaBO_2$, is converted into borax by CO_2 as follows:

$$4NaBO_2 + CO_2 \rightleftharpoons Na_2CO_3 + Na_2B_4O_7$$
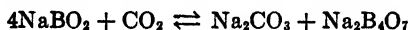
If the hydrolysis of the first equation proceeds completely from left to right the total osmotic pressure of the boron-containing compounds or ions will be *increased* in the ratio 4:1, while if the second reaction proceeds completely from left to right the total osmotic pressure of its boron-containing compounds or ions will be *reduced* in the ratio of 1:4. If either reaction goes on only partially the osmotic pressure of the boron-containing compounds will be increased, or reduced, in some intermediate ratio. Thus a fairly extensive change in the osmotic pressure per atom of boron can be caused, either by hydrolysis or by combination with CO_2, and these reactions provide an example of a chemical mechanism possibly analogous to the case of hemoglobin. It is conceivable that a similar change of osmotic pressure might be brought about in hemoglobin by varying concentra-

tions of Na· or H·, and that the mechanism of such a change might be explained by the assumption that hemoglobin is a salt of a weak acid, hydrolyzed in a manner similar to borax.

SUMMARY.

The S-shape of the O_2 absorption curve of blood can be explained by the hypothesis that the osmotic pressure of hemoglobin in blood is only $\dfrac{1}{n}$ part of the pressure of the O_2 with which it combines, where n has a value usually of rather less than 2.5. This reduction of the osmotic pressure of hemoglobin is probably analogous to similar effects occurring with weak acids, *e.g.* boric acid, in the presence of varying concentrations of hydrogen and basic ions, and described under the general heading of "hydrolysis."

The effects of acid and CO_2 upon the O_2 absorption curve of blood can be deduced from the hypothesis that the complex hemoglobin molecules $(Hb)_n$ and $(HbO_2)_n$ are like H_2CO_3, electrolytically dissociated in the forms

$$H\,(Hb)_n \rightleftarrows H· + (Hb)'_n$$
$$H\,(HbO_2)_n \rightleftarrows H· + (HbO_2)'_n$$
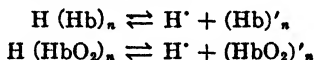
the degree of dissociation of the latter being far greater than of the former.

The same hypothesis accounts quantitatively for the extra CO_2 taken up by reduced in excess of that by oxygenated blood. At high CO_2 pressures, and at a given C_H, the taking up of a given volume V of O_2 results in a diminished CO_2 absorption equal to $\dfrac{V}{n}$.

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A MICRO METHOD FOR THE ESTIMATION OF AMMONIA IN BLOOD AND IN ORGANIC FLUIDS.

By K. L. GAD-ANDRESEN.

(From the Laboratory of Zoophysiology, University of Copenhagen, Copenhagen, Denmark.)

In an earlier publication[1] dealing with a micro method for the estimation of urea in blood, I also described a micro method for the estimation of ammonia in blood and in organic secretions. For the determination of the ammonia two analyses are made; in the first the total urea plus ammonia is determined; and in the second the urea alone after the ammonia has been removed by evaporating the blood in vacuum. The difference between the two analyses gives the ammonia content.

In the urea method itself the blood proteins are precipitated with 0.01 N acetic acid to which sodium acetate is added as a buffer. The precipitate is filtered off and the urea in the filtrate is estimated by means of its decomposition with hypobromite. The nitrogen produced is measured in Krogh's micro respirometer and calculated with the aid of Krogh's formula in terms of c.mm. When this figure is multiplied by 1.256 a correction for the weight of nitrogen is obtained in mg. and can, if desired, be found in terms of urea. For the purpose of this determination the micro respirometer is provided with ellipsoid bottles of 10 to 15 cc. capacity, fitted with ground glass stoppers on which are sealed small containers for the hypobromite. The determinations require from 0.10 to 0.15 cc. of blood and the error is ±0.5 mg. per 100 cc. Since the value for the ammonia by this method is obtained as the difference between two such estimations, both subject to error, the possible error on the ammonia estimation itself is relatively high.

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[1] Gad-Andresen, K. L., *Biochem. Z.*, 1919, xcix

As it is well known from the work of Henriques and Christiansen,[2] the amount of ammonia in blood is very small and is probably constant at about the value 0.25 mg. per 100 cc., a figure of about the same magnitude as the error in the determination. Estimations by this method, therefore, give positive results only when the ammonia content is abnormally high. The method was worked out at the time because I felt that my urea method would be more accurate if the urea and ammonia were analyzed separately. But when Henriques and Christiansen's work appeared, it seemed that further study in the interest of the accuracy of the urea method was unnecessary; the ammonia correction of 0.25 mg. per 100 cc. could simply be subtracted from the total found in order to get the true urea value.

A series of ammonia estimations were, however, made in part on human and in part on ox blood. The highest value found was 0.8 mg. per 100 cc. but as a rule none was found, indicating the amount present to be less than 0.5 mg. per 100 cc.—in good agreement with the results of Henriques and Christiansen with their macro method.

If 0.1 to 0.15 cc. of blood be used the method can indicate only, whether the ammonia content is about normal or not. Greater accuracy might be obtained with larger amounts of blood but in its present form the method can only be used with small amounts of blood. Were larger quantities of blood used, correspondingly larger quantities of fluid would be required for coagulating the blood and washing the precipitate, the size of containers must needs be increased, and hence the change in the manometer would be reduced. The requirements for an exact method are evidently that the amounts used for analysis be large and the value for the container as small as possible.

The method to be described meets these requirements. The amount of blood used is 1 cc. and the volume of the container is reduced to about 10 cc. The ammonia is estimated directly. After addition of borate (Sörensen[3]), the blood is evaporated to dryness in the apparatus presently to be described. The ammonia

[2] Henriques, V., and Christiansen, E., *Biochem. Z.*, 1917, lxxviii, 165; lxxx, 297.

[3] Sörensen, S. P. L., Emzymstudien. Mitteilungen vom Carlsberg Laboratorium, 1909, viii, pt. 1.

volatilized is driven by means of an air current over into the bottle of the micro respiration apparatus which contains weak sulfuric acid. It is thereupon estimated in the same way as in the urea method described above, the ammonia being decomposed by hypobromite, and the volume of nitrogen is determined.

The evaporating apparatus is very simple. It consists of a glass tube 25 cm. $\times$ 10 mm. provided with a bulb, as shown in Fig. 1, in which the blood and borate are mixed. The air current which is introduced at the other end through a small bent glass tube and rubber stopper first passes through a wash bottle containing concentrated sulfuric acid, which not only frees it from
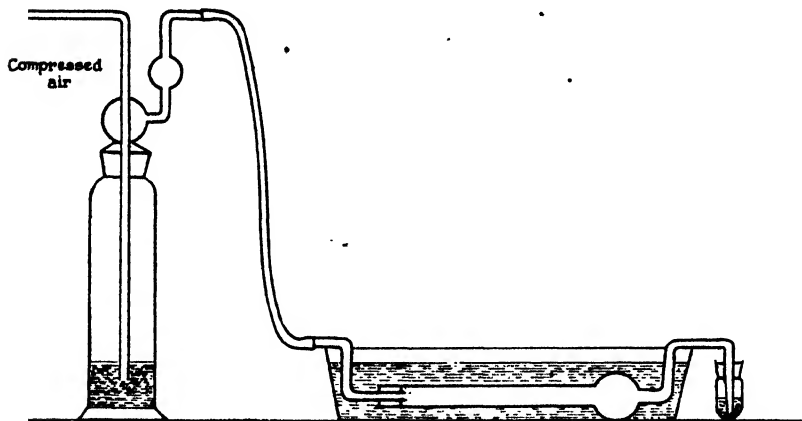
Fig. 1.

ammonia but also dries it and thus greatly increases the rate at which the blood is evaporated. The other end of the evaporating apparatus is drawn out into a narrow tube and bent as shown, so that it will dip under the surface of the sulfuric acid in the analysis bottle. During evaporation the apparatus is placed in a thermostat at about 25°. A temperature exceeding 30° must be avoided because, as it is well known, urea is then converted into ammonia.

A hypobromite solution of constant composition must be used, controlled, and corrected for by a Kjeldahl determination, as Krogh[4, 5] showed for urea determinations. Her formula is used for

[4] Krogh, M., *Z. physiol. Chem.*, 1913, lxxxiv, 379.
[5] Krogh, M., *Deutsch. Arch. klin. Med.*, 1916, cxx, 272

this purpose, the solution containing 1 cc. of bromine to 100 cc. of 2 N sodium hydroxide. The ratio between the nitrogen as determined by a Kjeldahl estimation and as found by the present method gives the factor for correction.

In the present instance the factor was found as follows:

An ammonium sulfate solution containing 0.4715 gm. per 100 cc. of water yielded 0.101 gm. of nitrogen. A series of estimations with the micro respirometer on the same solution gave an average value of 0.0919 gm. of nitrogen per 100 cc. and the correction was, therefore, $\dfrac{0.101}{0.0919} = 1.09$. The estimations with the micro respirometer were made under precisely the same conditions as those for blood. 0.1 cc. of the ammonium sulfate solution was measured into the analysis bottle in which had been placed 0.5 cc. of 0.2 N sulfuric acid and 1.9 cc. of water, making in all 2.5 cc. of fluid of the same acidity as under the conditions of blood ammonia analysis. The procedure was precisely as described in the urea method.

The ammonia estimations on blood are done as follows: 1 cc. of blood is accurately measured out and placed in the bulb of the apparatus, into which has been previously put 0.1 cc. of borate (9 cc. of borate + 1 cc. of NaOH). The stopper is inserted and the tube rotated so that blood and borax are thoroughly mixed. The apparatus is then tilted so that the blood flows towards the end of the tube where the air current enters. Care must be taken not to get any of the blood into the narrow connecting tube because when the air current is opened it may then be carried over into the acid and ruin the determination. The blood is distributed over the entire surface of the tube and the latter is then placed in the water bath and connected up with the bottle of the micro respiration apparatus, in which has been placed 0.5 cc. of 0.2 N H_2SO_4. The air current is cautiously opened and regulated so as to prevent the possibility of the material splashing out of the bottle. Evaporation to dryness is complete in about 30 minutes and the ammonia is estimated in the usual way in the micro respiration apparatus.

In order to facilitate the calculation of the evolved quantity of nitrogen it is to be noted that the same quantity of fluid is to be used in the analysis reservoir each time. This can be made so that the reservoir is tared and then, when the analysis is finished, weighs up with distilled water to a certain weight; for instance,

2.5 gm. This weighing must be made as correct as possible and must not differ at the utmost more than 0.10 gm. If care is taken that the volume of the analysis reservoir is not altered, the calculation of the analysis result is very simple, as the c.mm. of evolved nitrogen is multiplied by the proportion:

$$\frac{1.256 \times 1.09 \times 10^2}{V \times 10^6}$$

in which V signifies the quantity of blood used for the determination.

Analysis of ammonia both in normal blood and in the blood to which definite amounts of ammonia, as ammonium sulfate, have been added, are given in Table I.

TABLE I.

Estimations on Blood to Which Ammonia Has Been Added, Calculated as Ammonia Nitrogen per 100 Cc.

Double estimation directly on blood.

Blood.	Ammonia nitrogen per 100 cc.
	mg.
1	0.41 — 0.44
2	0.51 — 0.46
3	0.41 — 0.38
4	0.39 — 0.39

Blood 4 added 3.43 mg. of ammonia nitrogen per 100 cc.

Blood.	Ammonia nitrogen per 100 cc.		
	Found.	Average.	Calculated.
	mg.	*mg.*	*mg.*
8	3.86 — 3.84	3.85	3.82

Blood 4 added 5.36 mg. of ammonia nitrogen per 100 cc.

Ammonia nitrogen per 100 cc.		
Found.	Average.	Calculated.
mg.	*mg.*	*mg.*
5.78 — 5.81	5.79	5.75

The results show an average error of 0.03 mg. per 100 cc., but an error of 0.05 mg. may be taken as the limit.

Secretions as well as blood may be analyzed by this method, but it cannot be used for tissues, because, as I[6] have shown in an earlier paper, there is a rapid conversion of urea into ammonia after death. It may be prevented if the tissue is at once placed in alcohol cooled to $-20°$, but the employment of the alcohol will prevent an exact estimation with the micro respirometer.

I am obliged to Professor A. Krogh, the chief of the Zoophysiological Laboratory of Copenhagen for his kind attention to my work.

[6] Gad-Andresen, K. L., *J. Biol. Chem.*, 1919, xxxix, 267.

A MICRO UREASE METHOD FOR THE ESTIMATION OF UREA IN BLOOD, SECRETIONS, AND TISSUES.

By K. L. GAD-ANDRESEN.

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In the previous communication I have described a micro method for the estimation of ammonia in blood and in organic secretions. Utilizing the same principle, a micro urease method has been worked out for the estimation of urea in blood, tissues, and organic secretions. The procedure is exactly the same as that described under the ammonia method, except that in this case the urea is first converted into ammonia by means of urease anhydride, after which borate is added and the blood evaporated to dryness. The same correction is applied to the nitrogen found as in the ammonia method (that is, multiplication by the factor 1.09), and the calculation of results is quite the same.

The procedure estimates at the same time the preformed ammonia, but as this is very small and almost constantly 0.25 mg. per 100 cc., this figure may safely be used for subtraction to obtain the true urea value. If desired, however, or where the blood is abnormal, the ammonia may be determined separately and the correction found directly. As previously reported[1] I follow the procedure of Cullen and Van Slyke[2] in their modified macro urease method, using a solution of urease anhydride instead of the aqueous extract of the soy bean originally employed by Marshall.[3] The employment of urease anhydride is rendered safer if the time required to split the approximate amount of urea is previously determined. Otherwise one runs the risk that other nitrogen-containing substances present may be acted upon and

[1] Gad-Andresen, K. L., *J. Biol. Chem.*, 1922, li, 367.

[2] Cullen, G. E., and Van Slyke, D. D., *J. Biol. Chem.*, 1914, xix, 211.

[3] Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xiv, 283.

yield ammonia, as is the case when blood stands 12 hours with an aqueous extract of soy bean. For this standardization it is convenient to use 0.1 cc. of a 0.1 per cent urea solution in water together with 0.2 cc. of a 5 per cent urease anhydride solution which also contains 0.6 per cent primary potassium phosphate (KH_2PO_4). In this way an extra measurement is avoided and besides the urease anhydride solution is more stable when it contains primary potassium phosphate. The urease anhydride is placed in the bulb of the apparatus described in the ammonia method, the urea is added to it, and after the apparatus has been connected it is rotated in such a way that the two solutions are mixed. It is then immersed in a water bath at 38–40°C. for about 20 minutes, after which time 0.01 cc. of borate (9 cc. of borate + 1 cc. of sodium hydroxide) is added, and the tube is rotated in such a way that the solutions are well mixed. The procedure is then the same as described under the ammonia method except that the evaporation is carried out with the apparatus immersed in the water bath and is completed in about 15 minutes when the air current is properly regulated. The ammonia is then estimated in the micro respirometer in the usual way.

In order to make the calculation of the volume of nitrogen evolved easier, the same amount of fluid should be employed each time in the bottle, by previously taring the latter. The urease which I have used splits the urea up in the course of 20 minutes.

The number of c.mm. of nitrogen found is converted into mg. of nitrogen per 100 cc. of solution by multiplying by:

$$\frac{1.256 \times 1.09 \times 10^2}{0.10 \times 10^6}\cdot$$

In this formula the figure 0.10 represents the amount of urea solution taken.

Urea estimations on blood are made as follows: From 0.05 to 0.1 cc. of blood is measured in a micro pipette and at once inserted into the bulb of the apparatus, in which 0.2 cc. of the urease solution has been placed, previously standardized, as already described with respect to the time needed for the complete conversion of the urea. The apparatus is placed in the water bath at 38–40°C., and after the necessary time has elapsed 0.01 cc. of

borate is added and the evaporation carried out exactly as has been described.

Urea estimations in secretions are carried out in exactly the same way. Because of the relatively large urea content, it is necessary to dilute urine for estimation in the ratio 1:50 and take 0.1 cc. for an estimation. It is, of course, necessary to estimate the ammonia content of the urine as well, since this is not constant as in blood and organic secretions, and individual corrections must be made. For this ammonia estimation 0.05 to 0.10 cc. of urine is used instead of 1 cc. as in the case of blood and other secretions.

For tissue estimations I use 0.05 to 0.1 gm. of tissue, weighed into a tared weighing bottle, and which is macerated in a small

TABLE I.

Urea nitrogen per 100 cc.

Blood.	Found.	Average.	Calculated.	Liquid analyzed.
			mg.	*0.1 per cent urea solution*
1	47.2 — 47.0 — 46.7	47.0	46.7	
2	15.6 — 15.9 — 16.1	·15.9		Ox blood.
	63.1 — 62.8	62.9	62.6	Blood 2 plus 0.10 gm. urea per 100 cc.
3	7.3 — 7.7	7.5		Frog blood.
4	7.5 — 8.0	7.8		Frog bile.
5	7.7 — 7.5	7.6		Frog muscle.

agate mortar before it is put into the apparatus. With such small quantities, grinding with sand is unnecessary and the latter if used will later cause difficulty in the estimation. The crushing one can facilitate by cutting the tissue into very small bits, and making it as fine as possible with the aid of the mortar. By means of a small card and a glass spatula it can be transferred into the bulb of the apparatus. The mortar is rinsed twice with 0.25 cc. of water, which is transferred to the bulb with a micro pipette. Finally, 0.2 cc. of the urease anhydride is added. Care must be taken that the tissue is completely covered with the fluid so that the urease can act on the urea present and on this account more water must occasionally be added.

Because of the time required for the urea to diffuse from the tissue the reaction with the urease is slower than in the case of

blood. When the tissue has been finely divided one can count on an extra 10 minutes being sufficient for the purpose.

In Table I some results are given which will indicate the accuracy of the method.

As the results show, the average error is 0.4 mg. per 100 cc. The greatest error in individual determinations is 0.5 mg. per 100 cm. which indicates that the micro urease method as here given is more accurate than the macro urease method in which there is an error of 1 to 2 mg. per 100 cc.

SUMMARY.

A micro urease method is given for the estimation of urea in blood, secretions, and tissue. The method gives directly the content of urea plus ammonia, but the ammonia is allowed for by a correction of 0.25 mg. per 100 cc. or can, if desired, be separately determined.

The limit of error is 0.5 mg. per 100 cc.

A SYSTEM OF BLOOD ANALYSIS.

A NEW ÇOLORIMETRIC METHOD FOR THE DETERMINATION OF THE AMINO-ACID NITROGEN IN BLOOD.

By OTTO FOLIN.

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INTRODUCTION.

Next to the urea nitrogen the largest portion of the non-protein nitrogen in blood is represented by the nitrogen of the amino-acids. It is manifestly undesirable to try to obtain figures for this large portion by "calculation" as is frequently done. The undetermined nitrogen obtained by calculation contains not only amino-acids, but contains also nitrogenous products approaching the polypeptides in composition and character. These products must receive further study and it is partly with the idea of paving the way for the investigations of these unknown products that I have endeavored to find a simple direct method for the determination of the amino-acids in the tungstic acid blood filtrates.

The amino-acid work described in this paper was begun in the fall of 1919 and a preliminary report was made at the annual meeting of the American Society of Biological Chemists the following December. I gradually became convinced, however, that the method as we then used it was not sufficiently reliable to merit publication and it is only now after a great deal of further study that I venture to describe and recommend the method. The work was begun as a joint investigation by myself and Dr. Wu; but he was not able to remain until it was finished.

My immediate and main object was to find a method for the determination of the amino-acid nitrogen in blood, but I have,

of course, also had in mind the determination of amino-acids in urine; and since the preliminary critical work involved the testing of all available amino-acids present in proteins a suitable method if obtained would necessarily also be suitable for the determination of such amino-acids in many other materials such as gastric contents, milk, meat extracts, protein hydrolysis mixtures of various kinds, and innumerable food and medicinal preparations, representing various stages of decomposed (hydrolyzed) protein materials.

In this connection I must express my great obligation to Drs. H. D. Dakin, C. O. Johns, J. H. Koessler, R. B. Gibson, V. C. Myers, D. W. Wilson, J. C. Bock, and S. P. L. Sörensen for valuable gifts of pure amino-acid preparations.

While looking through the literature for hints as to what kind of a substance might be able to yield the required color reaction I came upon an old observation of Wurster's to the effect that ordinary o-quinone gives a color reaction with amino-acids and with proteins. This quinone was soon found to be unsuitable, but the observation served as a basis for the investigation of all o-quinones which we could obtain; and through the courtesy of Professor E. P. Kohler we were able to test out a considerable number. None seemed to be as promising, however, as β-naphthoquinone-sulfonic acid, the sodium salt of which happened to be among our own stock of chemicals. β-naphthoquinone-sulfonic acid has long been known to give a bright red precipitate with aniline. It has also been used as a color reagent for the quantitative estimation of indole with which it gives a blue color; but the fact that it also gives a very intense and decidedly stable color with amino-acids appears to have so far escaped discovery. It may be mentioned that the reaction with aniline has been shown to be due to the sulfonic acid group, whereas the reaction with amino-acids is presumably due to the o-quinone group, since ordinary o-quinone and many of its derivatives show a tendency to give such reactions. The great majority of the quinones are quite unsuitable, either because of their own intense color or because of their lack of suitable solubility. The fact that the quinones have so much color of their own would seem *a priori* almost to destroy their usefulness for quantitative work, especially in connection with the analysis of blood filtrates, 10 cc. of which contain less than 0.1 mg. of

amino-acid nitrogen. This aspect of the problem has indeed been a great stumbling block and it is only by a happy combination of circumstances that I have been able completely to overcome the difficulty.

A colorimetric estimation of amino-acids has been sought for by others, notably by Harding who made use of "ninhydrin" originally introduced by Abderhalden as a test for pregnancy. This reagent is difficult to procure. According to Harding it, moreover, fails in the presence of urea and thus could not be made available for the investigation of blood or urine except on the basis of more or less complicated and uncertain isolation processes. β-naphthoquinone-sulfonic acid on the other hand does not give a color with any of the main nitrogenous waste products, except ammonia, and ammonia is very easily removed. Urea, uric acid, creatinine, creatine, or hippuric acid give no color. As already indicated indole does give a blue color. The color obtained with amino-acids is red and, moreover, indole, at least in reasonable amounts, does not give a color with β-naphthoquinone-sulfonic acid under the conditions used for the production of the color reaction with amino-acids. The blue indole reaction is obtained only in the presence of strong alkalies (sodium hydroxide).

Theoretically it is, of course, rather unfortunate that β-naphthoquinone-sulfonic acid should give a reaction with ammonia. Theoretical considerations are of minor importance, however, in a problem of this sort, because that problem can probably never be solved on the basis of any perfectly specific reaction. The amino-acids are too numerous and too different in chemical constitution for that. It seems to me that the investigators of the ninhydrin reaction have rather overemphasized the fact that ammonium salts give the reaction. The ammonia in blood is certainly so insignificant as to be of no consequence in connection with the determination of amino-acid nitrogen. In the case of urine the situation is different, and there are doubtless other materials where the reaction with ammonia (and amines) cannot be left out of consideration.

Discussion of the Reagents Used for the Determination of the Amino-Acid Nitrogen in Blood.

1. *Standard Amino-Acid Solution.*—The standard solution of amino-acid used in blood analysis should contain 0,07 mg. of nitrogen per cc. Where there is a possibility of one's wanting to make other amino-acid determinations it is more convenient to make a stock solution containing 0.1 mg. per cc. The solution is made with 0.1 N hydrochloric acid, and 0.2 per cent of sodium benzoate. All the amino-acids (except perhaps tryptophane) seem to keep indefinitely. From the stock solution containing 0.1 mg. of nitrogen per cc. the blood standard is made by diluting 70 cc. with 0.1 N hydrochloric acid to a volume of 100 cc.

The question of which amino-acid to use in the preparation of the standard solution depends, of course, on which can be obtained in pure condition. Any one of the following amino-acids can be used: glycine, glutamic acid, leucine, phenylalanine, tyrosine. To this list I might add aspartic acid and cystine. Cystine has the advantage that it can be prepared so easily in strictly pure condition. The color obtained from cystine is, however, a trifle more yellow than the color obtained from the other amino-acids mentioned and in addition it is rather less dependable than the others with respect to the intensity of the color. I have, in fact, had much trouble about adjusting the conditions so as to get the full value from cystine. If the conditions are not right it will be too weak by from 5 to 7 or even 10 per cent. The objection to aspartic acid is that it is the slowest of all the amino-acids in relation to the development of the color. For miscellaneous work at least, this is a distinct drawback. Glutamic acid also tends to be rather slow and for this reason is the least desirable of the amino-acids mentioned as serviceable for the preparation of standard solutions. Personally I now use glycine exclusively as the standard and my only reason for not recommending this amino-acid alone is that the American supply of the pure article is rather uncertain. This objection is perhaps now not so important as it was a short time ago. It is scarcely safe to depend on nitrogen determinations to check up the purity of glycine because the impurities in it contain nitrogen. Glycine, as well as a number of other amino-acids, can easily be recrystallized by dissolving

in water and precipitating by the addition of 0.5 to 1 volume of alcohol.

2. *Special Sodium Carbonate Solution.*—The color reaction between amino-acids and β-naphthoquinone-sulfonic acid takes place very slowly in neutral solutions. The stronger the alkalinity, up to a certain point, the more rapidly does the color develop. The different amino-acids do not show the same acceleration, however, to definite increases in alkalinity, and the chromophoric reagent (the quinone) is more rapidly destroyed when the alkalinity is increased, giving rise to deep colored decomposition products. The finding of the right degree of alkalinity has, therefore, been rather difficult. Sodium hydroxide, magnesium hydroxide, mixtures of trisodium and disodium phosphate, disodium phosphate alone, sodium pyrophosphate, borax, sodium bicarbonate, and sodium carbonate—all in various amounts— have been tested out. Particular attention was given to borax, after it had been found that it has a special solvent effect on the quinone and also protects it from spontaneous decomposition, but it was not possible to obtain so good results with borax as could be obtained with other alkalies.

The required carbonate solution is made as follows: 50 cc. of approximately saturated solution are diluted to a volume of 500 cc. The strength of the resulting solution is determined by titrating 20 cc. of 0.1 N hydrochloric acid with the carbonate and with methyl red as indicator. On the basis of the titration value thus obtained the carbonate solution is diluted so that 8.5 cc. are equivalent to 20 cc. of 0.1 N acid. The carbonate solution is about 1 per cent. The titration method given serves as a guide for duplicating the carbonate solution which I am using

The correct degree of alkalinity is obtained when 1 cc. of this sodium carbonate solution is added to 1 cc. of amino-acid solution which at the same time is a 0.1 N solution of hydrochloric acid. The alkalinity is, therefore, represented by a mixture of carbonate and bicarbonate. A drop of phenolphthalein solution should always be used, is in fact indispensable when working with amino-acid solutions of unknown and variable acidity. It is desirable that the alkalinity in the different solutions, the standard and the unknowns, should be approximately the same, but there is no need of trying to make them exactly equal.

3. Fresh 0.5 Per Cent Solution of the Sodium Salt of β-Naphtho-quinone-Sulfonic Acid.—For the preparation of this quinone in pure form see page 386. Enough of this compound for several thousand amino-acid determinations can be made in the course of two mornings. In solution β-naphthoquinone-sulfonic acid is gradually decomposed and the solution becomes visibly darker in the course of a few hours, particularly if it is not kept in the dark. For this reason only freshly prepared solutions should be used. For practical work I find it convenient to charge a series of clean specimen tubes with 100 or with 500 mg. of the dry quinone in roughly powdered form. The samples need not be weighed any more accurately than can be done on a small torsion balance, because a variation either way of 5 per cent makes no difference. Transfer 100 mg. of the quinone to a small flask, add 20 cc. of water, and shake. Complete solution is obtained almost at once. For miscellaneous amino-acid determinations when 0.1 mg. of nitrogen is the standard, 3 cc. of the reagent are taken; for 5 cc. of blood filtrate only 1 cc.

4. Special Acetic Acid-Acetate Solution.—Dilute 100 cc. of 50 per cent acetic acid with an equal volume of 5 per cent sodium acetate solution. The presence of the sodium acetate in this solution serves two purposes. It serves to increase unmistakably the color of the quinone-amino-acid derivative, and it retards very much the onset of turbidity due to the liberation of sulfur from the added sodium thiosulfate. Both of these results are due to the weakened acidity of the acetic acid.

5. A 4 Per Cent Solution of Sodium Thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$). —This solution is used to destroy the surplus quinone remaining after the full color obtainable from the amino-acids has developed. It destroys the surplus color of the quinone and under the conditions prescribed has no effect on the colored quinone-amino-acid derivative, at least during the first 1 or 2 hours. Nor do the colored solutions become turbid from liberated sulfur within the first 2 hour period.

Before I had discovered how to destroy the surplus reagent with thiosulfate the proportionality of the color obtained with different amounts of the same amino-acid was not at all satisfactory and the amount of reagent which could be used was limited by the fact that with every increase the proportionality became poorer.

The proportionality was also unduly variable throughout the earlier stages of the investigation because of the influence of light on the spontaneous decompositions. The color from 0.066 mg. of amino-acid nitrogen would give a reading of 25.5 to 27 mm. instead of 30 mm., the theoretical value, when the standard representing 0.1 mg. was set at 20 mm. Since learning how to destroy the surplus quinone I have used as much as 50 mg. for 0.1 mg: of nitrogen, but as nothing is gained by so doing I have returned to the amount found adequate in the earlier work; namely, 15 mg. of quinone for 0.1 mg. of nitrogen. For the destruction of the quinone eight times as much of sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) should be used.

Description of the Determination.

As the number of practical determinations applicable to the tungstic acid filtrate increases, it becomes increasingly important not to use more of the filtrate for any one determination than is necessary for reliable results. 5 cc. of the filtrate are adequate for the amino-acid determination, but if the filtrate is abundant 10 cc. make the process perhaps a little more convenient.

Transfer to a test-tube (capacity 30 to 35 cc.) 1 cc. of the standard acid glycine solution representing 0.07 mg. of nitrogen and add 3 cc. of water. To another similar test-tube add 5 cc. of the blood filtrate. Add 1 drop of 0.25 per cent phenolphthalein solution to each. Add 1 cc. of the 1 per cent sodium carbonate solution to the standard and then add carefully, drop by drop, enough of the sodium carbonate solution to the blood filtrate until it has approximately the same pink color as the standard (3 or 4 small drops are usually required).

Add another 5 cc. of water to the standard; the volume of the standard is to be twice that of the blood filtrate. Then prepare a fresh 0.5 per cent solution of the sodium salt of β-naphthoquinone-sulfonic acid; add 2 cc. of this solution to the standard and 1 cc. to the blood filtrate. Shake a little to make the solutions uniform and set them aside in a completely dark cupboard and leave them there till the following day; that is, for 19 to 30 hours.

At the end of the time specified add the acetic acid-acetate solution—2 cc. to the standard and 1 cc. to the blood filtrate.

After the acetic acid has been added (never before) add the thiosulfate solution—2 cc. to the standard and 1 cc. to the blood filtrate. Finally add with a "blood pipette" 14 cc. of water to the standard giving a volume of 30 cc. and add 7 cc. of water to the blood filtrate (final volume 15 cc.). Mix and make the color comparison setting the standard at 20 mm.

For this work it is more convenient to use test-tubes graduated at 15 and at 30 cc. In order not to multiply unnecessarily the kinds of graduated test-tubes needed in blood analysis I now use exclusively the test-tubes, graduated at 25 cc., which are used for the blood urea determination. A second graduation is made at 15 cc. for the blood filtrates and the test-tube containing the standard is first diluted to the 25 cc. mark and an additional 5 cc. of water is then added before the final mixing.

20 divided by the colorimetric reading in mm., times 7; or 140 divided by the colorimetric reading, gives the amino-acid nitrogen in milligrams per 100 cc. of blood (5 cc. of the blood filtrate corresponds to 0.5 cc. of blood).

If 10 cc. of blood filtrate can be spared for the amino-acid determination it is more convenient to make use of test-tubes graduated only at 25 cc.

Transfer 1 cc. of the standard amino-acid solution containing 1 cc. of 0.1 N acid and 0.07 mg. of nitrogen to one such test-tube and to it add 8 cc. of water. To another such test-tube add 10 cc. of blood filtrate. Add 1 drop of phenolphthalein to each. Add 1 cc. of the special 1 per cent sodium carbonate to the standard, and to the blood filtrate add the carbonate solution, drop by drop, until the color obtained matches approximately that of the standard. Add 2 cc. of a freshly prepared 0.5 per cent β-naphthoquinone solution to each, mix, and set aside over night in a perfectly dark place. The next day add first 2 cc. of the acetic acid-acetate solution and then 2 cc. of the 4 per cent thiosulfate solution to each test-tube. Dilute to the 25 cc. mark, mix, and make the color comparison.

140 divided by the colorimetric reading obtained gives, as before, the amino-acid nitrogen in milligrams per 100 cc. of blood.

The second process is more satisfactory than the first in the case of bloods which are unusually low in amino-acid nitrogen, because in such cases, when the unknown is much weaker than the standard the match in color is not very good, the weak one being

more yellow. This difference in shade is not due to something other than amino-acids in blood, for the same phenomenon is encountered when working with unequal, but unusually weak solution of pure amino-acids.

It may become necessary with the first method to use a second standard containing 0.05 mg. of nitrogen, as I do in connection with the determination of the amino-acids in blood plasma.

The merits and the shortcomings of the method are adequately shown by the figures recorded in Table I (except in the case

TABLE I.

Showing the Colorimetric Readings (in Mm.) of Different Amino-Acids with the Color from Glycine as the Standard.

Amino-acid.	15 mg. reagent.			25 mg. reagent.		
	1 hr.	3 hrs.	21 hrs.	1 hr.	3 hrs.	21 hrs.
Glycine..........................	20	20	20	20	20	20
⅔ glycine..........................	30.7	30	30.3	30.7	30.7	29.7
Alanine..........................	18.4	18	18.3	18.1	18.5	18
Aspartic acid.....................	30	24	20.3	26	21.5	20
Glutamic acid....................	22.6	20.3	20	20.7	20.2	20
Cystine..........................	25.2	24	20	23.7	23.1	19.7
Valine...........................	20.5	20.6	20.6	21.7	21.2	20
Phenylalanine..	20	19.4	20	20	20	19.6
Tyrosine.........................	20	20	20.3	20.3	20.3	19.6
Lysine (0.1 N)..	21.5	20	19.6	20	20	20
Histidine ($\frac{N}{3}$)	21.7	20.3	20	22.5	21	19.6
Proline..........................	17	15	16	17	15	16
Tryptophane (0.5 N)..............	17.5	16.5	17	17	16.5	16.5

of arginine) and there is little need for added discussion. Glycine corresponding to 0.1 mg. of N was used as the standard, and another sample of glycine representing 0.066 mg. was introduced to show the proportionality of the reaction. All the other amino-acid solutions were made to contain the same amount of nitrogen as the standard, except in the case of histidine, which reacts with one-third of its nitrogen, and tryptophane, which has been taken to react with one-half. The values obtained for arginine are decidedly unsatisfactory. Because of the peculiarly low values obtained with arginine I was long in doubt about the purity of the

material, but substantially the same values were found for all the arginine preparations including a fine sample of arginine carbonate obtained from Professor S. P. L. Sörensen in Copenhagen and several samples of arginine phosphotungstate prepared by myself (from protamine). In all of these the color obtained corresponds only to about 16 per cent of the total arginine nitrogen. The reason why arginine behaves in such a peculiar manner with this amino-acid reagent must be left for further investigations.

These color comparisons were made as follows: The aminoacids were dissolved in 0.1 N hydrochloric acid and were so made as to contain 0.1 mg. of nitrogen (or active nitrogen) per cc. To 1 cc. of each solution in a graduated test-tube were added 1 drop of phenolphthalein solution, 1 cc. of the 1 per cent sodium carbonate solution, 7 cc. (or 5 cc.) of water, and 3 cc. (or 5 cc.) of fresh 0.5 per cent amino-acid reagent. The test-tubes were then placed in a dark closet. At the end of 1, 3, or 21 hours the contents were acidified by the addition of 1 cc. of special acetic acid-acetate solution. 3 cc. (or 5 cc.) of the thiosulfate solution were then added; the solutions were made up to volume (25 cc.); and the color comparisons were made (within 1 hour).

Detailed Description of Preparation of the Amino-Acid Reagent.

The process described below for the preparation of strictly pure β-naphthoquinone-sulfonic acid sodium salt is the outcome of a great many trial experiments. No effort has been spared to make the preparation simple as well as reliable. From the list of needed chemicals and from the different steps in the process enumerated, the preparation may seem a rather formidable undertaking but in actual practice it will be found that the amount of work involved is not very large. The description has purposely been made so explicit that a person with limited chemical experience cannot go astray, except by not following the directions. I would warn against introducing variations or modifications of any kind, for I have tried a great number of plausible short cuts and variations, only to find that they had no merit.

The following chemicals are needed for the preparation of 75 to 90 gm. of the pure reagent:

Cold 10 per cent sulfuric acid 1,000 cc.
Concentrated nitric acid 100 cc.
    "   hydrochloric acid 500 cc.
10 per cent sodium hydroxide solution 300 cc.
10 "   "   "   chloride solution 2,000 cc.
Bromine 1 cc.
Sodium nitrite 50 gm.
    "   nitrate 100 gm.
    "   sulfite 50 gm.
    " ' bisulfite 100 gm.
Borax 400 gm.
Resublimed β-naphthol 100 gm.
Alcohol about 2,000 cc.
Ether about 200 cc.
Ice 1,000 gm.

1. Transfer 100 gm. of β-naphthol to a liter beaker; add 300 cc. of 10 per cent sodium hydroxide solution and stir with a glass rod until complete solution is obtained (10 to 15 minutes).

2. Transfer 50 to 55 gm. of sodium nitrite to a 4 liter beaker; add 600 cc. of water and shake until solution is obtained (3 to 5 minutes).

3. Pour the alkaline β-naphthol solution into the 4 liter beaker holding the nitrite solution, and rinse with about 100 cc. of water.

4. Add 800 gm. of crushed ice to the naphthol-nitrite mixture.

5. Fill a 200 cc. cylinder with cold dilute (10 per cent) sulfuric acid and pour it slowly down one side of the beaker, while stirring vigorously and continuously with a heavy glass rod. Continue the stirring for 1 to 2 minutes after all the acid in the cylinder has been added. Then fill the cylinder again and add this in the same way. Repeat this addition of dilute sulfuric acid, 200 cc. at a time, until 800 cc. have been added. The additions should be continued until the mixture in the beaker gives a distinct and *permanent* acid reaction with Congo red paper (time 15 to 20 minutes).

A yellow precipitate begins to form with the first addition of acid and increases in quantity until the whole mixture becomes a semisolid paste. The precipitate will have a slight greenish tint; if it is distinctly green the conditions are not right and a less good yield is obtained. Let stand for 1 hour after the last of the acid has been added. It is important not to omit this detail because without such a period of standing much unchanged

β-naphthol remains in the mixture and is encountered when try-
ing to dissolve the precipitate in sulfites.   Longer standing does
no harm, but is superfluous.

6. Filter through a 20 cm. Buchner funnel with *moderate*
suction and wash with about 1,500 cc. of cold water.

7. Transfer the precipitate (nitroso-β-naphthol) by blowing,
to a large evaporating dish and sprinkle over it 100 gm. of sodium
bisulfite and 50 gm. of sodium sulfite; stir with a spoon (glass
or enameled ware).   An extremely soluble bisulfite addition
product is formed and the mixture becomes liquid.   Filter imme-
diately on a Buchner funnel (diameter 12 fo 15 cm.) through a
double layer of good (quantitative) filter paper, from the small
amount of black residue and wash with a little water.

8. Transfer the filtrate and washings at once (to avoid excessive
darkening) to a 5 liter flask or wide mouth (colored) bottle, con-
taining 2,000 cc. of water and 500 cc. of concentrated hydrochloric
acid.   Cover with a funnel and one or two watch-glasses and let
stand in a *dark* closet for about 36 hours (24 hours is not quite
enough).   The whole flask becomes filled with a network of white
needles which carry down a little dark and pink matter as impuri-
ties.   The greater the exposure to light the more of the dark
decomposition products will be formed.

Filter on a 20 cm. Buchner funnel with moderate suction and
wash with about 2 liters of cold water.

9. Blow the precipitate (1-amino-2-naphthol-4-sulfonic acid)
to a large filter paper and from there transfer it to a large beaker
(3 to 4 liter capacity).   Sprinkle over the precipitate 100 gm.
of sodium nitrate.   Dilute 100 cc. of concentrated nitric acid with
350 cc. of water and pour the whole of this lukewarm dilute acid
into the beaker.   Reaction begins immediately and nitric oxide
fumes begin to come off.   Leave without stirring for 10 minutes
while the greater part of the reaction takes place.   Then stir
thoroughly for a few minutes and let stand for another 20 to 30
minutes.

If no visible reaction takes place on adding the nitric acid the
cause is probably to be found in the presence of sodium carbonate
in the sodium nitrates used.   Even samples of sodium nitrate
which are labeled "The Standard of Purity" may contain consid-
erable amounts of carbonates.   If no reaction takes place add a
little (1 to 5 cc.) of concentrated nitric acid.

At the end of about half an hour filter on a Buchner funnel (diameter 15 cm.) and wash with about 1,000 cc. of 10 per cent sodium chloride solution.

The light brown product on the funnel is the desired sodium salt of 1-2-4-naphthoquinone-sulfonic acid. But it is not pure. It contains dark-colored decomposition products, and also traces of ammonia. Considerable ammonia is formed (from the amino group) during the oxidation with the nitric acid, but because of the large amount of sodium nitrate present only traces of the ammonia are carried down as the ammonium salt of the quinone. By recrystallization under the conditions described in the next section (Section 10) the product is freed from all disturbing impurities.

10. Transfer the moist precipitate to a large porcelain dish. Add 200 gm. of powdered borax and 450 cc. of water. Mix with a pestle until all but a few flakes of the quinone have dissolved. Filter through a good quality (quantitative) filter paper on a 10 cm. Buchner funnel from the surplus borax and a little undissolved black residue. Because of the latter the filtration is apt to be rather slow and it is better not to apply too strong a suction. Wash with 100 to 150 cc. of water.

While the filtration is proceeding, transfer 850 cc. of 95 per cent alcohol and 150 cc. of concentrated hydrochloric acid to a Florence flask. Cover the mouth of the flask with a beaker and cool under running water.

Transfer the quinone-borax filtrate to a 4 liter beaker. Add a few drops of liquid bromine to the cooled acid-alcohol. Shake until the bromine is dissolved and then pour the resulting straw-yellow solution into the quinone-borax mixture and stir quickly and vigorously for a few moments so as to secure complete mixing. Let stand for 5 minutes. The quinone has all come down at the end of this time.

Filter on a Buchner funnel (diameter 15 cc.) and wash with 700 to 800 cc. of 10 per cent solution of sodium chloride.

This one recrystallization is adequate as far as the color of the product is concerned, but it still contains some ammonia. The recrystallization must, therefore, be repeated once more. This final recrystallization is conducted in the same way, except with the difference that the washing of the quinone on the Buchner

funnel with sodium chloride is omitted, and for it is substituted the washing, first with 300 to 400 cc. of alcohol and finally, with about 200 cc. of ether. 75 to 90 gm. of perfectly pure quinone is thus obtained.

The purification process described above is the outcome of a large number of experiments directed toward obtaining this quinone free from colored decomposition products. The compound cannot be recrystallized from water alone, because each time it is dissolved there is some decomposition and when precipitation is made (usually by the addition of sodium chloride) the decomposition products adhere to the compound and it thus becomes darker than it was before.

Before I discovered the protecting and dissolving action of borax on β-naphthoquinone-sulfonic acid I had prepared it in pure form by the following much more expensive and wasteful process.

To 100 gm. of powdered quinone in a 2 liter beaker were added: first, 1,000 cc. of bromine water; and then, with stirring, 700 cc. of boiling water. The warm solution was at once filtered through a large folded filter into a 4 liter flask.

To the filtrate was added 2,500 cc. of 95 per cent alcohol followed immediately by 150 gm. of powdered sodium chloride. The mixture was shaken hard under running water until cold (10 minutes) and was filtered and washed with alcohol and ether.

A second similar recrystallization (but omitting the first filtration) gave a perfectly pure product. As this process required the use of 6 liters of alcohol and involved considerable losses of quinone the borax process described above is manifestly much superior.

The following tests for the purity of the sodium salt of 1-2-4-naphthoquinone-sulfonic acid will be found useful.

Color.—Prepare a fresh 1 per cent solution of the quinone in water and compare its color with that of a 0.5 N solution of potassium bichromate with the latter set at 20 mm. in the colorimeter. The quinone solution will read 26 to 27 mm.

Colored Decomposition Products.—Transfer 2 cc. of the fresh 1 per cent quinone solution to a test-tube. Dilute to a volume of 25 cc. . Add: first, 1 cc. of 50 per cent acetic acid; and then, 1 cc. of 15 per cent sodium thiosulfate solution. The solution will bleach in the course of a few seconds so completely that it is only by looking through the length of the tube that a faint yellow shade is visible.

Ammonia.—Transfer 10 cc. of the solution to a small flask. Add about 2 gm. of permutit and shake gently for 3 to 4 minutes. Decant and wash with distilled water four or five times, until all of the yellow color is gone.

Then add to the permutit powder a few drops of 10 per cent sodium hydroxide solution, 5 cc. of water, and 5 cc. of Nessler's solution. No color is produced.

A COLORIMETRIC DETERMINATION OF THE AMINO-ACID NITROGEN IN NORMAL URINE.

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For the colorimetric determination of the amino-acid nitrogen in normal urine by the method described in the preceding paper[1] it is only necessary to first remove the ammonia.

The removal of the ammonia is easily accomplished by means of permutit. Urine differs from blood filtrates in that the concentration of the ammonia to be removed varies within very wide limits and the amino-acid concentration is also subject to very large variations. It is, therefore, not always easy to tell just how much urine should be taken. The amino-acid excretion is substantially independent of the volume of the urine and, since the normal excretion is usually between 4 and 12 mg. per hour, one can practically always get suitable amounts for the color reaction.

The process is as follows:

Dilute from 5 to 25 cc. of urine to a volume of 25 cc. in a 50 cc. Erlenmeyer flask. Add 2 to 3 gm. of permutit[2] and agitate very gently, but continuously for 5 minutes. Decant the supernatant urine into another 50 cc. flask. Again add 2 to 3 gm. of permutit, and shake as before for 5 minutes. By this double extraction with permutit every trace of ammonia is removed. Decant the supernatant urine into a flask or test-tube. It may be a little turbid, but this fact does not interfere with the determination.

To test-tubes graduated at 25 cc. add 1, 2, and 3 cc., respectively, of a standard glycocoll solution in 0.1 N hydrochloric acid plus

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[1] Folin, O., *J. Biol. Chem.*, 1922, li, 377.

[2] The special permutit to be used is that prepared by the Permutit Company of New York. The product, I believe, is now obtained from the leading American dealers in chemicals. It is, of course, essential to know that the product is active and does take ammonia.

0.2 per cent of sodium benzoate. This standard solution should contain 0.1 mg. of glycocoll nitrogen per cc. To these tubes add 1, 2, and 3 cc., respectively, of the special 1 per cent sodium carbonate solution described in the preceding paper[1] (1 cc. of sodium carbonate for each cc. of 0.1 N hydrochloric acid present). Dilute the contents of each test-tube to a volume of 10 cc.

Transfer 5 cc. of the ammonia-free (usually diluted) urine to another test-tube graduated at 25 cc. Add 1 cc. of 0.1 N hydrochloric acid and 1 cc. of the 1 per cent sodium carbonate solution. Dilute to 10 cc. Dissolve 250 mg. of the amino-acid reagent in 50 cc. of water, and add 5 cc. of this solution to each standard and to the unknown urine.

Mix and set in a dark place over night. It is often advisable to take out the test-tubes and inspect them after they have stood 10 to 15 minutes. If the test-tube containing the urine appears much darker than the darkest standard, as may happen, especially in connection with experiments planned to produce excessive amino-acid excretion, then it is, of course, necessary to start another sample of the urine, taking only 1, 2, or 3 cc. and treating it in the same way as the first sample, not omitting to provide for a final volume of 15 cc.

The following day the standard and the unknown or unknowns are first acidified by the addition of 1 cc. of the special 25 per cent acetic acid-acetate solution. To each are then added 5 cc. of the 4 per cent sodium thiosulfate solution. The contents of all the tubes are diluted to a volume of 25 cc. and, after mixing, the color of the unknown is read against that of the standard having most nearly the same intensity of color.

For the calculation it is, of course, essential to know which standard is used, and the actual volume of undiluted urine taken for the determination.

THE RETENTION AND DISTRIBUTION OF AMINO-ACIDS WITH ESPECIAL REFERENCE TO THE UREA FORMATION.

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CONTENTS.

INTRODUCTION.

In connection with our recently described investigations on the transportation, retention, and excretion of carbohydrates, we have made a very large number of analyses of blood and urine bearing on the simultaneous fluctuations of nitrogenous constituents such as amino-acids, urea, and creatine. Since one of the questions involved was that raised by Falta concerning the permeability of human blood corpuscles it seemed well worth while to settle this question for the nitrogenous products as well as for sugar— a settlement which we believed that our method for separating plasma enabled us to furnish. Our amino-acid determinations were made by the new colorimetric methods described in the two preceding papers.[1]

The extent to which the amino-acid excretion is influenced by fluctuations in the amino-acid concentration in the blood represents a practically untouched problem. While one cannot expect to find any really constant relationship between the concentration

[1] Folin, O., *J. Biol. Chem.*, 1922, li, 377, 393.

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in the blood and the concentration in the urine or the hourly excretion, it is important to have data on the normal simultaneous variations in blood and urine, because these data can at least serve as a guide and check by which to judge alleged pathological deviations. At present it is for example impossible to tell what significance, if any, can properly be ascribed to the "excessive" amino-acid excretion which is obtained after giving glycocoll or gelatin to patients in whom the deamination power of the liver is believed to be reduced.

We shall discuss this test for the liver function in connection with some of our experiments.

Critical and Experimental Examination of the Hypothesis that Deamination and Urea Formation are Localized in the Liver.

The localized character of the deamination process by which the absorbed amino-acids lose their nitrogen and yield urea was definitely disproved by Folin and Denis[2] in a series of articles published in 1912. By determinations of the urea and the non-protein nitrogen in the blood during the absorption of single pure amino-acids, glycine, alanine, aspartic acid, and asparagine they proved that deamination and urea formation did not occur, at least to any material extent, while the amino-acids passed through the walls of the intestine or through the liver. On the other hand, they did obtain increases in the non-protein nitrogen of the blood, and also of the tissues, in amounts sufficient to account for approximately the whole of the absorbed nitrogen. They obtained substantially the same results whether they used a single amino-acid, or pancreatic digestion mixtures which could have recombined into protein material during the absorption. These results definitely excluded both deamination and protein regeneration as features of amino-acid absorption and were interpreted as excluding equally definitely the doctrine that the urea formation is localized in the liver.

The experimental results of Folin and Denis were abundantly confirmed the following year (1913) by Van Slyke,[3] and as his

[2] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 87; 1912, xii, 141.
[3] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399; 1913–14, xvi, 197, 213.

results were obtained by his new gasometric method for the determination of amino-acids he naturally regarded them as even more conclusive.

In one fundamentally important point Van Slyke's conclusions differed from the conclusions drawn by Folin and Denis. He revived once more the old doctrine that the formation of urea from the nitrogen of amino-acids is a special function of the liver.

The experimental results which Van Slyke interpreted as evidence in favor of the liver as a special "locus" for the urea formation are very important. Unless the interpretation adopted by Van Slyke is correct those results tend to prove quite the opposite, namely that the liver does not even transform the nitrogen of the amino-acids which it does take up into urea, but soon lets go of those amino-acids for distribution to the other tissues. The experimental results in question are as follows:

The liver takes up amino-acids more rapidly and extensively than the muscles just as might be expected from its enormous blood supply. Within 3 hours after the injection of the amino-acids into the blood the surplus stored in the liver has already left it, whereas the amino-acids absorbed by the muscles have then scarcely begun to diminish. Is this extraordinary disappearance of the amino-acids from the liver due to urea formation in that organ or is it due to the same cause by which it at first received an excessive proportion of the amino-acids, namely the abundant supply of blood, of blood from which the muscles and other tissues have in the meantime abstracted much of the amino-acids? In our opinion the last named process is the more plausible explanation of Van Slyke's interesting data.

The vitality of the old concept that the liver represents a specialized locus for the urea formation is such that it has received over and over again the benefit of the doubts, in attempts to explain data which could possibly be construed as having any bearing on the origin of urea. The worthlessness of all the older evidence purporting to show that the urea was formed more or less exclusively in the liver was clearly brought out by Münzer and Winterberg[4] in 1893. The theory was again revived as a corollary to the hypothesis that the amino-acids lose their nitrogen during

[4] Münzer, E., and Winterberg, H., *Arch. exp. Path. u. Pharmakol.*, 1893–94, xxxiii, 164.

the process of absorption; and almost immediately after this hypothesis, with its corollary, had been destroyed by the experiments of Folin and Denis the theory of the predominant urea formation in the liver was again revived by Van Slyke. Van Slyke does not claim that his results prove that urea is formed mainly in the liver, but in adopting such urea formation as the most plausible explanation of his experimental results, he has again given a new lease of life to this old and popular theory.

In Van Slyke's interpretation the old theory is formulated with precision and in terms of the modern knowledge of the chemistry and the metabolism of the proteins. The surplus of amino-acids stored in the liver, in consequence of the excessive absorption, is speedily deaminized and the nitrogen removed in the form of urea, and, later, the amino-acids stored in the muscles and other tissues are likewise transferred to the liver and there subjected to the same process.[5] As we cannot accept this interpretation we purpose to discuss the available data bearing on the subject.

The experimental conditions of Van Slyke as well as those of Folin and Denis led to excessive temporary storage of amino-acids both in the liver and in the muscles. It is possible that in consequence of such excessive retentions the muscles received more material than they could utilize, and that the surplus under such conditions is transferred to the liver as soon as this organ has relieved itself of its own excessive supply and is capable of receiving the new influx. It seems to us at least equally plausible, however, that a process substantially the reverse of that thus described by Van Slyke takes place under such conditions.

The liver, partly by its location, partly because of its enormous blood supply, acts as a buffer or safety valve in relation to a large and rapid influx of amino-acids from the digestive tract or as a result of amino-acid injections. The absorption by other tissues, especially the muscles, is not so rapid, because all the capillaries through which the muscles receive materials are not equally and simultaneously open (Krogh), hence all the muscles are not filled equally fast; but for the same reason the muscular tissues, taken as a whole, can continue to take up materials for a considerable time. The muscles thus continue to abstract amino-acids from the blood, but the loss in the latter is made good by corres-

[5] Van Slyke, D. D., *The Harvey Lectures*, 1915–16, xi, 146.

ponding acquisitions from the liver, until after 3 hours, according to Van Slyke's results, the liver has been freed from its surplus.

For evidence on the problem one must turn to the data available on the urea production. Van Slyke's hypothesis requires immediate and rapid urea formation while the liver is loaded, practically to full capacity, with amino-acids. At the end of 3 hours when the liver has only its normal amount and the muscles are still nearly at their maximum content of amino-acids the urea formation should certainly be reduced to a very small fraction of what would take place while the liver is full. Van Slyke recognizes the importance of immediate and extensive urea formation for the maintenance of his theory and in his Harvey lecture,[5] given 3 years after the publication of his paper on the locus of the urea formation, he refers to experiments purporting to show that the urea formation begins the very moment that amino-acids begin to reach the liver.

These experiments are lacking in the precision necessary to prove so fine a point. They consisted of heavy meat feeding to dogs and finding that the urea content of the blood had already begun to rise, when the gastric contents were beginning to enter the duodenum, the exact moment of which was determined by means of x-rays. But Folin and Lyman[6] showed (1912) that absorption from the stomach takes place and leads to an unmistakable increase of the urea in the blood. Such absorption must manifestly have occurred in Van Slyke's meat feeding experiments, not only on the basis of the digestion products formed in the stomach, but also on the basis of the ammonia and urea present in the meat.

More definite data on the urea formation associated with the amino-acid absorption are contained in the papers of Folin and Denis. Take for example Experiment 3 of the first paper.[2] After a 6 minute absorption period following the injection of glycocoll into the intestine the non-protein nitrogen of the blood had risen from 30 mg., the original value, to 34 mg., and the urea nitrogen had risen only 1 mg.—from 18 to 19; and during the following 12 minutes the non-protein nitrogen rose from 34 to 47 mg. while the urea nitrogen increased only from 19 to 22 mg. The very

[6] Folin, O., and Lyman, H., J. Biol. Chem., 1912, xii, 259; 1912–13, xiii, 389.

large increase in the non-protein nitrogen in this blood showed that the liver had been flooded with glycocoll and that the muscles also had been offered a more abundant supply than they were able to take up. Yet the urea nitrogen content of the blood was increased by only 3 mg., and this notwithstanding the fact that the exit of urea through the kidneys was prevented by ligatures. The validity of these results and other similar ones have been accepted by Van Slyke and others, including Abderhalden, as showing that deamination of amino-acids does not take place while they pass through the walls of the intestine. But no one has attempted to explain why they do not also show that no material deamination and urea formation occurs while they pass through the liver. Whether the given, relatively small, increase in the urea nitrogen of the blood was due to the liver, the muscles, or both, is of course debatable, but the figures certainly show that the muscles had had an abundant opportunity to take part in the urea formation that did occur.

To prove that the liver is even the predominant place for deamination and urea formation it is necessary to show that during the early stages of absorption of amino-acids the increase in the urea content of the blood *precedes* the increase in its content of amino-acids. If the increase in the amino-acid content precedes that of the urea as in the experiment cited this does not conclusively prove that the liver is not the special seat of the formation, because the urea-forming process may be very much slower than the absorption process under such unusual conditions. But such a condition certainly leaves intact the view that the liver has not developed any special powers of deamination.

In order to obtain more convincing evidence as to whether the liver has the function ascribed to it by Van Slyke it is necesary to provide for a more moderate speed of absorption than prevailed in the experiments of Van Slyke or of Folin and Denis. And on the basis of the further refinements in the analytical technique which has been obtained since 1912, it is now possible to obtain unmistakable results under much more normal conditions. As all are now agreed that the greater part of the protein is absorbed as simple amino-acids and that the amino-acids, in so far as they pass the liver, are absorbed by all the other tissues, the whole problem has really narrowed down to the one question of whether

the absorbed nitrogen does or does not pass out of the liver in the form of urea.

In Table I we give the results obtained from one substantial meal taken, without any preceding breakfast, at 11.45 a.m. to 12 m. We have two experiments of this sort made at the same time on two different persons, but, as the results are substantially identical, only one is recorded. The meal consisted of three boiled eggs, a pint of milk, together with bread, butter, and coffee. From the figures given it will be seen that from such a perfectly ordinary mixed meal there was obtained a definite, unmistakable increase in the amino-acid content of the blood. Within $1\frac{1}{2}$ hours the amino-acid content of the plasma rose from 5.2 to 5.8 mg. per 100 cc. In the course of the next hour it rose to 6.4 mg., and $5\frac{1}{2}$ hours after the ingestion it had again sunk to very near the fasting value.

The figures cited will naturally raise the question as to whether the amino-acid method is really capable of finding so small a difference as only 1 mg. of amino-acid nitrogen per 100 cc. The difference amounts to 20 per cent, and we are certain that the figures, relative to each other, are correct. The amino-acid determinations were all started and finished together. It will further be noted that the amino-acid excretion with the *urine* confirms the values obtained for the blood. As we have other different experiments it is not necessary to dwell too much on this one. It is given mostly for the purpose of showing that an ordinary meal, not excessively rich in protein, is accompanied by a slowly increasing concentration of the amino-acids in the blood, an important fact since we know that accompanying that increase there must be a large increase in the amino-acid content of the muscles.

Table II gives the figures for blood and urine obtained after taking 135 gm. of (Knox's) gelatin, dissolved in 900 cc. of water. In taking so large a dose of protein we have, of course, exceeded the strictly normal conditions, for man, but the results obtained are also correspondingly striking. It should be explained that the subject, H.B-d., had eaten freely, and probably rather too liberally, for 1 or 2 days before and that the fasting values for the blood at the beginning of the experiment were, therefore, unusually high.

TABLE I.

Subject H. B-d.   Age 34 years.   Weight 90 kilos.

Time.		Blood per 100 cc.				Urine per hour.				Remarks.
Mar. 20, 1921.		Amino-acid N.	Urea N.	Rest N.	Total non-protein N.	Volume.	Amino-acid N.	Urea N.	Total N.	Fasting. Urinated at 9.30 a.m.
a.m.		*mg.*	*mg.*	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*	*mg.*	
	Blood.	6.2	10.4	11.8	28.4					
11.42	Plasma.	**5.2**	**11.4**	**5.7**	**22.3**	80	7.2	431	571	
	Corpuscles.	8.0	8.6	22.6	39.2					
11.45 a.m. to 12.00 m.										Lunch: 3 eggs, 450 cc. milk, bread, butter, and coffee.
p.m.										
	Blood.	6.6	11.0							
1.23	Plasma.	**5.8**	**11.8**	**5.2**	**22.8**					
	Corpuscles.	8.0	9.6							
2.25						51	9.3	470	572	
	Blood.	7.2	10.8	12.4	30.4					
2.33	Plasma.	**6.4**	**12.0**	**6.0**	**24.4**					
	Corpuscles.	8.6	8.7	23.7	41.1					
3.47						43	8.9	392	491	
	Blood.	7.4	11.0	15.2	33.6					
4.08	Plasma.	**6.4**	**13.2**	**6.4**	**26.0**					
	Corpuscles.	9.2	7.1	31.0	47.2					
5.00						64	9.8	520	644	
	Blood.	6.4	12.1	13.5	32.0					
5.42	Plasma.	**5.6**	**13.4**	**6.0**	**25.0**					
	Corpuscles.	7.8	9.7	27.0	44.5					
6.10						57	6.9	488	572	

TABLE II.

Subject H. B-d.   Age 34 years.   Weight 90 kilos.

Time.		Blood per 100 cc.				Urine per hour.				Remarks
Feb. 7-8, 1921.	·	Amino-acid N.	Urea N.	Rest N.	Total non-protein N.	Volume.	Amino-acid N.	Urea N.	Total N.	Fasting. Urinated at 9.00 a.m.
p m.		*mg.*	*mg.*	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*	*mg.*	
	Blood.	6.7	15.2	17.5	39.4					
2.25	Plasma.	5.5	17.3	6.2	29.0	27	10.0	403	465	2.55 to 3.20 p.m. Took 135 gm. gelatin and 900 cc. water.
	Corpuscles.	8.4	12.2	33.8	54.4					
	Blood.	7.6	14.2	17.6	39.4					Fasted during the whole experiment.
4.00	Plasma.	9.4	16.0	8.2	33.6					
	Corpuscles.	5.1	11.7	30.6	47.4					
4.37						39	9.3	459	518	
	Blood.	10.7	15.2	18.9	44.8					
4.52	Plasma.	11.0	14.2	14.2	39.4					
	Corpuscles.	10.2	16.6	25.6	52.4					
6.03						39	43.0	540	651	
	Blood.	11.7	18.9	17.9	48.5					
6.10	Plasma.	7.2	20.2	13.6	41.0					
	Corpuscles.	13.5	16.9	29.8	60.2					
7.15						77	96.0	962	1,158	
10.34						82	63.0	1,095	1,327	
	Blood.	7.0	24.6	18.9	50.5					
10.55	Plasma.	6.8	25.0	11.4	43.2					
	Corpuscles.	7.4	24.0	30.4	61.8					
a.m. 9.00						30	11.0	756	783	

In this case the amino-acid content of the plasma increased 100 per cent in 2 hours, rising from 5.5 to 11 mg. Gelatin is believed to be the most easily digested protein material and our results are in harmony with that view, because within 3 hours the amino content of the plasma had already fallen from the high point, 11 mg., to 7.2 mg. It is by no means certain, however, that gelatin taken in large doses does not in part escape complete digestion. Our figures for the undetermined (rest) nitrogen are quite high enough to permit the conclusion that some materials of a peptide character were absorbed.

The most interesting facts about this experiment are the fluctuations of the amino-acids and the urea. In our opinion they definitely prove that the liver has no specialized function in connection with deamination and subsequent urea formation. Here we have a 100 per cent increase in the amino-acid nitrogen of the blood without any increase in the urea content of the same, accompanied in fact by a slight diminution in the concentration of the urea. The urea formation clearly begins to gather force only after the incoming tide of the amino-acids has begun to subside. During the first 2 hours while the amino-acid nitrogen rose from 5.5 to 11 mg. the urea nitrogen sank from 17.3 to 14.2 mg. Then during the next hour and a quarter, while the amino-acid nitrogen sank to 7.2 mg., the urea nitrogen rose from 14.2 to 20.2 mg. From that point on there are only further reductions in the level of the amino-acids, but the concentration of the urea nitrogen increases still further, and 8 hours after the gelatin ingestion it stands at 25 mg. in the plasma.

The figures for the urea nitrogen or the total nitrogen of the urine tell the same story as the figures for the blood. During the first 2 hour period, while the amino-acid accumulation in the blood was approaching its maximum, the urea nitrogen excretion (per hour) rose only from 403 to 459 mg. At the end of about 3 hours, a period including the first real beginning of active urea accumulation in the blood, the urea nitrogen of the urine is still only 540 mg. per hour. At the end of this period the urea excretion jumps in a single hour from a rate of 540 mg. of urea nitrogen to one of 962 mg. per hour. And during the following 3 hours, beginning about 4 hours after the gelatin ingestion, urea nitrogen is excreted at the rate of 1,095 mg. per hour.

While we, for the sake of conciseness, have concentrated the discussion of the alleged localized urea formation on the gelatin experiment, the reader is asked to examine Tables I to VI. They all show that the *predominant* increase in the non-protein nitrogen of the plasma during the early stages of absorption is associated mainly with an increase in the amino-acid nitrogen—and the later stages with increases in the urea nitrogen. As a check on the results note also that in Table VIII, where we are dealing with carbohydrate absorption, no such increases are to be found.

It is perhaps also worth while to point out that the results have been obtained with normal human subjects, and under conditions which are as nearly normal as it is possible to make them.

As far as we can see there is now nothing more that need be said concerning the old tradition that one special function of the liver is to split off amino nitrogen and convert it into urea. We therefore refrain from referring to the mass of other evidence showing that the urea production continues when the liver is virtually eliminated, damaged, or destroyed.

On Amino-Acid Excretion as a Test for Liver Function.

A few brief comments on the so called liver function tests in cirrhosis of the liver may not be superfluous. These tests consist usually of giving 25 gm. of glycocoll or 50 gm. of gelatin, and then determining the amino-acids, by the formol titration, and the total nitrogen in the 24 hour urine. It is held that in advanced cirrhosis the percentage of nitrogen excreted as amino-acids is distinctly higher than the figures obtained under similar conditions from normal persons.

From the different tables included in this paper it will be seen that increases in the amino-acid excretion following the intake of nitrogenous food like gelatin or glycocoll does not last for more than a few hours. If the intake has been large the excretion will outlast the high level in the blood, presumably because the kidneys, like other tissues, have received excessive amounts. Even under these circumstances the excretion comes to an end long before the 24 hour period is over. 24 hour periods are, therefore, unsuitable for the test, especially if stress is laid on the percentage of the total nitrogen contained in the amino-acid fraction, for the urea excretion comes in later and obscures the

result. It might be thought that our findings concerning the special deamination power of the liver has completely removed the scientific foundation upon which the liver function test in question rested.

As a test for a non-existent liver function the test has no justification, but we are not prepared to say that the test in suitably modified form may not have some value. A substantial scientific foundation for the test is to be found in the fact, discovered by Van Slyke, that the normal liver does take up an excessive charge of amino-acids when the blood passing through it is rich in the same. It serves, as we have expressed it, as a safety valve or buffer, thus materially diminishing the concentration in the general circulation, and thereby also the elimination with the urine. But in cirrhosis the liver cells gradually disappear until practically nothing or very little is left. As the liver diminishes, its activity as a buffer is reduced and the consequence might well be an abnormally large excretion with the urine, especially during the first 4 to 6 hours. But the test is, of course, not a functional test. It is only a test of the mass of the remaining liver cells.

In Table III we give the analytical data for blood and urine following the intake of 25 gm. of glycocoll in 600 cc. of water. The amount is perhaps rather small for a person weighing 90 kilos but we believe that the figures obtained may, nevertheless, serve as a normal basis of comparison for the making of similar experiments on the subject suffering from cirrhosis of the liver, on the basis of 4 to 6 hour periods. It might be pointed out that for the amino-acid determinations in the blood the plasma figures are necessarily somewhat more instructive than those obtained with whole blood, because the latter include the more variable values obtained from the corpuscles. There is no justification for insisting on using the plasma, however; to do so requires the use of more blood, is more laborious, and does not involve the finding of anything which would escape detection by means of whole blood analysis.

From the figures for the blood taken at 11.26 a.m. it will be seen that at the end of only 20 minutes absorption there is an unmistakable increase in the amino-acid content of the plasma (and whole blood). At the end of 2 hours the amino-acid nitro-

TABLE III.

Subject H. B-d.   Age 34 years.   Weight 90 kilos.

Time.		Blood per 100 cc.				Urine per hour.				Remarks.
Jan. 11, 1922.		Amino-acid N.	Urea N.	Rest N.	Total non-protein N.	Volume.	Amino-acid N.	Urea + NH$_3$ – N.	Total N.	Fasting. Urinated at 9.00 a.m.
a.m.		mg.	mg.	mg.	mg.	cc.	mg.	mg.	mg.	
11.03	Blood.	6.05	10.3	13.7	30.0	28	5.8	452	474	
	Plasma.	4.8	13.0	7.0	24.8					
	Corpuscles.	8.1	6.0	24.3	38.4					
11.06										Took 25 gm. glycocoll and 600 cc. water.
11.26	Blood.	6.7	11.3	12.4	30.4					
	Plasma.	5.8	13.9	6.3	26 0					
	Corpuscles.	8.3	6.7	23.4	38.4					
p.m.										
12.08	Blood.	7.85	12.7	11.8	32.4					
	Plasma.	8.3	14.7	6 5	29 5					
	Corpuscles.	7.1	9.0	21.7	37.8					
12.33						49	15.8	585	641	
1.12	Blood.	8.0	11.8	11.2	31.0					
	Plasma.	7.8	12.5	9.8	30.1					
	Corpuscles.	8.4	10.4	13.6	32.4					
1.50						60	26 4	642	754	
4.07	Blood.	6 35	12.5	15.1	34.0					
	Plasma.	5.4	13.9	6.9	26.2					
	Corpuscles.	8.0	10.0	30.0	48.0					
4.34						34	13.7	478	543	

gen has risen from 4.8 to 7.8 mg., an increase of over 60 per cent. So far as the urea production is concerned it is not possible to draw definite conclusions on the basis of the small change found

here—and often encountered.   It must be remembered that the urea concentration in the blood is a resultant, partly of what the kidneys are doing and partly of the forces at work in the muscles. Folin and Denis always found the urea content of the muscles higher than in the blood, and no one has ever found less in the muscles than in the blood though Gad-Andresen[7] has recently found the same values for plasma and for muscles. For the urea production more definite results are, of course, obtainable from the urine.   It has been suggested (Folin and Denis[8]) that theoretically one might expect to obtain a more rapid production of urea from a given nitrogen absorption when the nitrogen is obtained from a single amino-acid, than when it is obtained from a mixture of many.   This suggestion is based on the hypothesis that the tissues probably tend to maintain amino-acid mixtures of more or less constant composition.   It is from this point of view at least suggestive that the nitrogen elimination from the ingested glycocoll has risen from 474 to 641 mg. per hour, $1\frac{1}{2}$ hours after the ingestion.   At 1.50 p.m., not quite 3 hours after the intake of the glycocoll, the maximum nitrogen elimination, 754 mg. per hour, is reached.

The hourly amino-acid excretion has risen from 5.8 to 26.4 mg. in about 3 hours, and it is still at a rather high level, 13.7 mg. per hour, at the end of 4 to 5 hours.

Significance of the Normal Excretion of Amino-Acids.

One of the questions which we have attempted to elucidate in this investigation is that of the origin and significance of the amino-acid nitrogen found in all normal human urines.   It is generally taken for granted that the excretion of amino-acids represents loss of material which the organism could use to advantage, if the loss could be prevented.   From a physiological standpoint it seems rather remarkable that the animal organism should be able to retain every trace of absorbed fat or glucose, yet is constantly losing amino-acids.   It occurred to us that the amino-acids of normal urine, like the carbohydrates of the same, might for the most part, at least represent denatured or more or

[7] Gad-Andresen, K. L., *Biochem. Z.*, 1921, cxvi, 266.
[8] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 141.

less foreign nitrogenous materials, rather than the ordinary amino-acids which we know as the main constituents of food proteins.

The increased amino-acid excretion obtained from the ingestion of 25 gm. of glycocoll (Table III) shows, however, that the amino-acids found in urine do indeed represent losses of ordinary usable amino-acids.

To get further light on this question we have studied the effects of casein. We chose casein because it is a protein in which there should be no unusable nitrogenous materials. Three of our experiments are worth recording. The first one, Table IV, is interesting not only because it illustrates the transient character which the amino-acid excretion may sometimes exhibit, but also because it proves, as clearly as the gelatin experiment, that urea formation does not accompany the passage of amino-acids through the liver.

The subject, Mr. G. Ph. R-s., received 12 gm. of nitrogen in the form of predigested casein, as well as 4 gm. of creatine. The creatine was included for a separate study and is mentioned here only to make the record accurate. Nearly 3 hours after the ingestion of the casein the subject received 100 gm. of pure glucose; this was also for a different purpose and need not be further referred to here. Mention must also be made of the fact that this subject, a student, was accustomed to eat heavily of meat products. His average 24 hour nitrogen was over 20 gm. and he was later instructed to reduce his consumption of protein. The high level of his nitrogen intake is also shown by the high fasting values for his blood urea and non-protein nitrogen. In 45 minutes the amino-acid nitrogen of the plasma rose from 5.3 to 9.2 mg.—an increase of 73 per cent, and is accompanied by practically no increase in the urea nitrogen of the plasma. The urea elimination, as it happens, fell from 806 mg. of nitrogen to 592 mg. per hour during the same period of maximum amino-acid absorption. At the end of $2\frac{1}{2}$ hours the deamination is extremely active as shown by the 27 mg. of urea nitrogen in the plasma and a urea nitrogen elimination of 1,140 mg. per hour.

The elimination of amino-acid nitrogen rose from the high fasting value of 9.6 mg. per hour to 19.5 mg.

The next two experiments (Tables V and VI) should be considered together, because the two subjects received substantially

TABLE IV.

Subject Mr. G. Ph. R-s.   Age 23 years.   Weight 90 kilos.

Time.		Blood per 100 cc.				Urine per hour.				Remarks.
Apr., 1921.		Amino-acid N.	Urea N.	Rest N.	Total non-protein N.	Volume.	Amino-acid N.	Urea N.	Total N.	Fasting. Urinated at 8.54 a.m.
a.m.		mg.	mg.	mg.	mg.	cc.	mg.	mg.	mg.	
11.00	Blood.	6.8	20.0	16.2	43.0					
	Plasma.	5.3	22.0	8.2	35.5	72	9.6	806	922	
	Corpuscles.	7.4	17.2	28.8	53.4					
11.05										Took 300 cc. predigested casein (12 gm. N) and 4 gm. crystalline creatine.
11.48	Blood.	9.6	21.0	18.7	49.3					
	Plasma.	9.2	23.0	10.0	42.2					
	Corpuscles.	10.1	18.2	30.7	59.0					
p.m.										
12.04						51	8.0	592	784	
1.15	Blood.	9.35	23.0	17.6	50.0					
	Plasma.	9.0	27.0	12.3	48.3					
	Corpuscles.	9.8	17.5	25.1	52.4					
1.30						88	19.5	1,140	1,445	Took 100 gm. glucose at 1.45 p.m.
3.05	Blood.	6.5	25.0	15.0	46.5					
	Plasma.	5.6	25.0	10.0	40.6					
	Corpuscles.	7.8	25.0	22.2	55.0					
3.30						80	13.1	1,115	1,379	

TABLE IV—*Concluded*.

Time.		Blood per 100 cc.				Urine per hour.				Remarks.
April, 1921.		Amino-acid N.	Urea N.	Rest N.	Total non-protein N.	Volume.	Amino-acid N.	Urea N.	Total N.	
p.m.		*mg.*	*mg.*	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*	*mg.*	
	Blood.	6.2	22.0	14.8	43.0					
4.45	Plasma.	**4.6**	**25.6**	9.2	**39.4**					
	Corpuscles.	8.3	17.2	22.2	47.7					
5.00						55	8.8	846	942	
	Blood.	5.9	23.5	11.6	41.0					
5.35	Plasma.	**4.65**	**25.0**	9.75	**39.4**					
	Corpuscles.	7.6	21.4	14.0	43.0					
6.00						60	8.9	976	1,060	

the same amount of casein (60 gm.), only in one case the casein was given in predigested form, and in the other it was taken in the form of a suspension. The taking of casein in the latter form proved unexpectedly difficult and, for the benefit of others who may wish to take or administer casein in a similar manner, we may say that it is almost hopeless to try to take much of it, as long as there remains dry casein dust floating on top of the suspension.

The two sets of figures recorded in Tables V and VI are not nearly so different as might have been expected. The predigested casein was, however, taken together with 120 gm. of glucose and the glucose may have modified, in a measure both the speed of absorption and the subsequent distribution of the amino-acids. From the predigested casein the amino-acid nitrogen rose in the plasma from 4.9 to 7.4 mg. The corresponding figures from the experiment with casein suspension are 5.05 and 7.6 mg. The amino-acid excretions rose from 4.9 to 9.3 mg. per hour in the former case and from 6.3 to 8.2 mg. in the latter. The amino-acid excretion is evidently independent of diuresis. The nitrogen

TABLE V.

Subject J. M. F-r.   Age 22 years.   Weight 72 kilos.

Time.		Blood per 100 cc.				Urine per hour.				Remarks.
Apr. 12, 1921.		Amino-acid N	Urea N.	Rest N.	Total non-protein N.	Volume.	Amino-acid N.	Urea N.	Total N.	Fasting. Urinated at 9.03 a.m.
a.m.		*mg.*	*mg.*	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*	*mg.*	
	Blood.	6.6	12.5	12.9	32.0					
10.52	Plasma.	4.9	12.5	9.2	26.6	36	4.9	371	469	
	Corpuscles.	9.0	12.5	18.3	39.8					
10.55										Took 250 cc. predigested casein (10 gm. N), 120 gm. glucose, and 500 cc. water.
	Blood.	8.1	13.9	16.4	38.4					
11.32	Plasma.	7.4	14.1	7.3	28.8					
	Corpuscles.	9.1	13.7	29.4	52.2					
11.53						44	5.0	464	576	
p.m.										
	Blood.	7.4	13.2	17.8	38.4					
12.55	Plasma.	6.3	14.3	10.4	31.0					
	Corpuscles.	9.1	11.5	29.4	50.0					
1.12						43	6.9	530	662	
	Blood.	5.9	14.7	12.7	33.3					
1.53	Plasma.	5.9	14.5	9.6	30.0					
	Corpuscles.	5.9	15.1	17.5	38.5					
2.52						41	9.3	564	734	
	Blood.	6.0	14.1	13.5	33.6					
3.47	Plasma.	4.6	14.4	9.3	28.3					
	Corpuscles.	8.4	13.6	20.8	42.8					

TABLE V—*Concluded.*

Time.		Blood per 100 cc.				Urine per hour.				Remarks.
Apr. 12, 1921.	.	Amino-acid N.	Urea N.	Rest N.	Total non-protein N.	Volume.	Amino-acid N.	Urea N.	Total N.	
p.m.		*mg.*	*mg*	*mg.*	*mg.*	*cc*	*mg.*	*mg.*	*mg.*	
4.25	.					46	8.3	566	730	
5.40	Blood.	6.2	15.0	14.3	35.5					
	Plasma.	4.0	15.7	11.2	30.9					
	Corpuscles.	9.9	13.9	19.4	43.2					
5.48						39	6.3	519	610	

excretion would seem at first glance to be more rapid from the undissolved than from the predigested casein, but the extraordinary diuresis obtained in the undissolved casein experiment may have influenced the nitrogen excretion to a material extent.

From all the experiments given, but especially those with casein and glycocoll, it is quite clear that losses of ordinary usable amino-acids is a normal occurrence, and that these losses are increased during and immediately following increases in the amino-acid content of the blood. There is nothing corresponding to the sharp glucose threshold at which the eliminations begin. In fact the retention and excretion phenomena associated with the amino-acids are more like those which we have found for galactose,[9] which has no threshold, than for anything else so far described. In the absence of a threshold or other sharp mechanism for the retention of the absorbed amino-acids one would expect to find a continuous excretion, a continuous loss, with the urine. And that is exactly what we do find. Under such conditions there should also be an increase in these losses, whenever there is an influx of amino-acids from the digestive tract sufficiently rapid to materially raise the amino-acid content of the blood. This happens after every fairly protein-rich meal.

[9] Folin, O., and Berglund, H., *J. Biol. Chem.*, 1922, li, 213.

TABLE VI.

Subject H. B-d.   Age 34 years.   Weight 90.kilos.

Time.		Blood per 100 cc.				Urine per hour.				Remarks.
		Amino-acid N.	Urea N.	Rest N.	Total non-protein N.	Volume.	Amino-acid N.	Urea + NH₃ – N.	Total N.	
Jan. 9, 1922.										Fasting. Urinated at 7.32 a.m.
p.m.		*mg.*	*mg.*	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*	*mg.*	
	Blood.	6.25	12.4	17.3	36.0					
12.25	Plasma.	5.05	13.0	8.6	26.6	23	6.3	360	445	
	Corpuscles.	8.1	11.6	31.3	51.0					
12.30 to 1.15										Took 60 gm. Hammarsten's casein and 600 cc. water.
1.52						190	5.7	606	670	
	Blood.	7.4	14.2	12.4	34.0					
2.03	Plasma.	7.25	13.6	8.6	29.5					
	Corpuscles.	7.65	15.0	18.3	41.0					
2.18						462	7.1	650	712	
	Blood.	8.15	15.2	12.6	36.0					
3.00	Plasma.	7.6	15.6	7.2	30.4					
	Corpuscles.	9.1	14.5	21.4	45.0					
3.46						57	8.2	635	650	
	Blood.	7.05	15.2	13.7	36.0					
4.55	Plasma.	6.15	16.6	8.2	31.0					
	Corpuscles.	8.5	13.0	22.5	44.0					
5.43						37	5.5	517	571	

Ambard and his associates have endeavored to expand the concept of a definite constant relationship between the concentration in the blood and the excretion, which was first propounded for urea, into a general law governing the excretion of all products.

Since the amino-acid excretion is not controlled by anything corresponding to the glucose threshold this law of Ambard's ought to apply to amino-acids. A candid examination of our tables will show, however, that nothing suggesting the existence of a constant, in the sense of Ambard, is to be found. Increased accumulations in the blood will be followed by increased excretions, but it is perfectly clear that it is not only the level in the blood plasma, but also the extent to which the tissues, including the kidneys, have been charged with amino-acids, which determine the rate of the excretion.

TABLE VII.

Normal Minimum, Maximum, and Average Blood Content of Non-Protein Nitrogenous Products (From 12 Young Men).

	Whole Blood per 100 cc.				Plasma per 100 cc.				Corpuscles per 100 cc.			
	Amino-acid N.	Urea N.	Undetermined rest N.	Total non-protein N.	Amino-acid N.	Urea N.	Undetermined rest N.	Total non-protein N.	Amino-acid N.	Urea N.	Undetermined rest N.	Total non-protein N.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
After a night's fast.												
Minimum	5.7	8.9	10.1	27.8	4.3	9.6	1.8	18.0	6.7	7.7	18.3	37.7
Maximum	7.8	15.2	17.5	39.4	6.2	17.3	11.5	30.0	10.7	13.2	33.8	55.0
Average	6.4	11.5	13.7	32.1	5.3	12.4	6.7	24.7	8.2	10.3	24.7	43.6
After carbohydrate intake.												
Minimum	4.9	8.0	6.4	21.0	3.5	9.2	1.8	17.0	5.9	5.2	9.0	24.8

While the amino-acid content of the blood may be raised by the intake of nitrogenous food, it does not continue to fall as a result of fasting. From an epileptic who had fasted 3 weeks, for therapeutic purposes, we obtained on the 20th day 5.0 mg. for plasma, 6.4 mg. for whole blood, and 7.8 mg. for the corpuscles—all given as for 100 cc. The average amino-acid nitrogen excretion for the same day was 2.4 mg. per hour, the total nitrogen excretion 336 mg. per hour.

The maximum, minimum, and average values obtained for the amino-acid nitrogen in the forenoon hours, when no breakfast is

TABLE VIII.

Subject Mr. D-n.   Age 22 years.   Weight 75 kilos.

Time.		Blood per 100 cc.				Urine per hour.				Remarks.
		Amino-acid N.	Urea N.	Rest N.	Total non-protein N.	Volume.	Amino-acid N.	Urea N.	Total N.	
Feb. 25, 1921.		*mg.*	*mg.*	*mg.*	*mg.*	*cc.*	*mg.*	*mg.*	*mg.*	Fasting. Urinated at 10.12 a.m.
	Blood.	5.8	10.3	13.9	30.0					
12.00 m.	Plasma.	4.3	10.6	9.3	24.2	50	5.7	387	486	Took 200 gm.
	Corpuscles.	7.8	9.9	20.0	37.7					glucose and 830 cc. water. at noon.
p.m										
	Blood.	5.7	10.3	15.4	31.4					
12.50	Plasma.	4.3	11.1	6.2	21.6					
	Corpuscles.	7.7	9.1	28.7	45.5					
1.30						111	2.9	327	356	
	Blood.	5.2	9.6	14.5	29.3					
1.50	Plasma.	3.5	10.6	5.6	19.7					
	Corpuscles.	7.7	8.2	27.3	43.2					
2.30						35	3.8	362	480	
	Blood.	4.9	10.0	11.1	26.0					
3.00	Plasma.	3.6	10.4	5.2	19.2					
	Corpuscles.	6.8	9.4	19.6	35.8					
4.00						29	4.9	376	485	
	Blood.	5.2	8.5	12.7	26.4					
4.35	Plasma.	3.7	9.2	5.3	18.2					
	Corpuscles.	7.3	7.5	23.0	37.8					
5.30						27	4.3	276	384	
	Blood.	5.0	8.8	13.0	26.8					
6.05	Plasma.	3.6	9.6	5.2	18.4					
	Corpuscles.	6.9	7.7	23.8	38.4					
6.50						76	3.7	382	453	Dinner at 7.00 p.m.
9.25						111	6.2	348	462	

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taken, together with similar figures for urea, undetermined nitrogen, and total non-protein nitrogen, are given in Table VII. This table also gives corresponding minimum values obtained under the influence of carbohydrate intake, not accompanied by any intake of protein (for 1 day).

In Table VIII are given data showing in detail the effect of carbohydrate intake on the composition of blood and urine. 200 gm. of pure glucose in 830 cc. of water were taken at noon, immediately after taking a sample of the subject's blood. As was to be expected from the absence of nitrogen intake and from the protein-sparing action of the sugar we meet here with small, but definite reductions in the different nitrogenous constituents in the blood. The amino-acid nitrogen sinks from 4.3 mg. per 100 cc. of plasma to 3.6 mg.; the urea nitrogen from 10.6 to 9.6 mg.; the undetermined nitrogen from 9.3 to 5.2 mg.; and the total non-protein nitrogen from 24.2 to 18.4 mg.

Distribution of the Normal Non-Protein Nitrogen.

In an earlier part of this paper we alluded to Falta's assertion that the blood corpuscles are free from non-protein nitrogenous constituents. We believe that the many simultaneous analyses of plasma and whole blood reported in this paper, and they represent only a small fraction of the total number made, prove beyond possibility of doubt that for the nitrogenous constituents human blood corpuscles are as permeable as we have previously shown them to be permeable for glucose.

So far as urea, the chief single nitrogenous compound, is concerned, our data point not only to permeability, but also to the production of urea in the corpuscles. It is not worth while to try to prove definitely this point, because there are so many variable factors involved, the effects of which cannot be accurately estimated, at least by the kind of experiments which we have made. It is according to our experience by no means uncommon to find, particularly after heavy protein intakes, samples of blood in which the urea concentration is higher in the corpuscles than in the plasma, even without making any allowance for the fact that the corpuscles contain much less water. This certainly suggests, but, of course, does not prove that the higher concentration is due to local production.

In all our tables will be found figures for the undetermined, or rest, nitrogen.   These figures are a little too high since they include the nitrogen of the uric acid, the creatinine, and the "creatine."   It will be seen that this unknown fraction is from two to five times as abundant in the corpuscles as in the plasma.   The nature, origin, and significance of the nitrogenous materials represented by this nitrogen must necessarily be left for future investigations to unravel.   We venture, however, to advance one surmise.

It is a well known fact that different blood protein precipitants give filtrates which, with the same blood, yield different amounts of non-protein nitrogen.   These differences must necessarily be due for the most part to how much of the unknown materials are taken by the precipitant.   If these unknowns are of the nature of proteins it may also be expected that the amino-acid nitrogen found will be influenced by the presence of these nitrogenous substances and the materially higher amino-acid nitrogen found in the corpuscles than in the plasma may in part be due to the richness of the corpuscles in the undetermined nitrogen.

The extraordinarily high values (20 mg.) obtained for the amino-acid nitrogen of the blood of birds and the fact that such blood is also enormously rich in undetermined nitrogen stand in need of some explanation.   It is on the basis of these facts that we venture to suggest that the undetermined nitrogenous materials are in part made up of histones.   It was from the corpuscles of birds that Kossel obtained histones and it is difficult to see how one can fail to obtain varying quantities of histones when working with the blood of birds.   It is precisely with such bloods that the different protein precipitants give the greatest differences in the amounts of non-protein nitrogen obtained.   The tungstic acid method for example gives 3 to 6 mg. less than the trichloroacetic acid or the m-phosphoric acid method.

Histones must be more abundant in bird's blood than in the blood of man or other mammals, or they would doubtless long since have been found in such bloods also; but it seems to us extremely probable that the undetermined nitrogen of human blood and other bloods, for which the corpuscles are largely responsible, is in very large part due to the presence of histones similar to those which have been obtained from the corpuscles of birds.

NOTE ON THE NECESSITY OF CHECKING UP THE QUALITY OF SODIUM TUNGSTATE USED IN THE SYSTEM OF BLOOD ANALYSIS.

By OTTO FOLIN.

(From the Biochemical Laboratory, Harvard Medical School, Boston.)

Because of the many inquiries received concerning different brands of sodium tungstate for use in precipitating the blood proteins it seems suitable to publish this brief note on the subject.

The statement made in the first paper[1] of "A system of blood analysis" that we obtained our sodium tungstate from the Primos Chemical Company was not intended to convey the impression that this company had been requested or had undertaken to supply the chemical according to our specifications. We simply found that their product was satisfactory and mentioned the fact. Being manufacturers, we thought that their brand would continue to be available. The firm has since ceased to make the product.

At that time we supposed that the only difficulty that might be expected in connection with sodium tungstate would be the presence of variable large excesses of sodium carbonate, and directions were given for titrating the alkali with 0.1 N acid, using phenolphthalein as indicator. We suggested that no tungstate should be used which contained more than 0.5 per cent of sodium carbonate. The amount of 0.1 N acid required for the titration of 10 cc. of 10 per cent tungstate solution should not exceed 0.4 cc. As a matter of experience, up to date, the sodium tungstates which show an alkaline reaction have come within this figure.

I have, however, since encountered sodium tungstates which show no alkaline reaction to phenolphthalein, though they are alkaline to litmus paper. These tungstates may be perfectly pure in the sense that they contain nothing but tungstates, yet

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[1] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 86.

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they will not yield a good uric acid reagent and they will give erroneous figures for blood analyses.

I deem it particularly important to call attention to this variety of sodium tungstate because almost any one might fail to note that they are unsuitable, since they will give perfectly clear blood filtrates. The titratable acidity of the filtrates will be excessive, however; the total nitrogen will be somewhat too low, and the uric acid and creatinine will be more or less completely removed from the filtrate.

These acid tungstates are usually so slightly soluble that one cannot readily make a 10 per cent solution in cold water, whereas the true sodium tungstate is soluble in 2 parts of water.

The acid tungstates need not be discarded. They can be made perfectly serviceable in the following manner:

Prepare 100 cc. of a 10 per cent solution in water, using heat if necessary. Cool. Titrate 10 cc. of the solution with N sodium hydroxide to a faint, but permanent, pink reaction, using phenolphthalein as indicator. The pink color should persist for at least 3 minutes after the last addition of alkali. The reason for this requirement is that the complex acid tungstates are only gradually and slowly decomposed by the slight excess of alkali added during the titration.

In making subsequent 10 per cent solutions of the tungstate add the amount of alkali indicated by the titration.

The essential point in this treatment is, of course, that the complex acid tungstates are easily converted into the simple salt, $Na_2WO_4:2H_2O$, by the addition of the required amount of alkali.

With the 10 per cent sodium tungstate so prepared and correct, 0.66 N sulfuric acid, we never fail to obtain blood filtrates suitable for subsequent analysis unless excessive amounts of potassium oxalate have been added to prevent clotting, and it is only because of such excess of oxalate that the addition of a little more sulfuric acid is occasionally required.

The preliminary conversion of the complex tungstates into the simple one by the addition of a suitable amount of alkali is also important for the preparation of the uric acid reagent and the phenol reagent. The more complex tungstic acids are seemingly not decomposed in acid solution; they, therefore, fail to combine with the phosphoric acid and the reagent will be weak.

COLORIMETRIC METHODS FOR THE SEPARATE DETERMINATION OF TYROSINE, TRYPTOPHANE, AND CYSTINE IN PROTEINS.

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(Received for publication, February 8, 1922.)

INTRODUCTION.

The colorimetric method for the estimation of tyrosine in proteins proposed by Folin and Denis[1] in 1912 has been shown to yield results which in many cases must be too high, because tryptophane and its decomposition products give the same blue reaction with the phenol reagent as does tyrosine. The method has been adversely criticized by a number of different investigators. Much of this criticism is manifestly based on inexperience in the field of colorimetry; but the error due to tryptophane, an error pointed out by Abderhalden,[2] tentatively admitted by Folin and Denis,[3] and later verified by Gortner and Holm[4] as well as by Thomas[5] has left us with a wide gap of uncertainty as to the true tyrosine content of many protein materials.

[1] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 239.
[2] Abderhalden, E., and Fuchs, D., *Z. physiol. Chem.*, 1913, lxxxiii, 468.
[3] Folin, O., and Denis, W., *J. Biol. Chem.*, 1913, xiv, 457.
[4] Gortner, R. A., and Holm, G. E., *J. Am. Chem. Soc.*, 1920, lxii, 678.
[5] Thomas, P., *Bull. Soc. chim. biol.*, 1921, iii, 198.

We shall not here discuss the extraordinary criticisms which some of the authors cited have expended upon the method. Gortner and Holm have tabulated nine different reasons why tyrosine cannot be estimated by means of the phenol reagent,[6] and, incidentally, have proved to their own satisfaction that the colorimetric sugar method of Folin and Wu must be worthless.

It is, of course, possible that definite sources of error in the colorimetric tyrosine method other than that due to tryptophane may be discovered soon or later. But a colorimetric analytical method yielding maximum figures must be considered very useful, at least as a check on the extremely uncertain, usually minimum, values obtained by direct isolation processes; and when a source of error in such a colorimetric method is found the discovery would seem to call for revision rather than for unqualified destruction.

The investigation here described was begun for the purpose of removing such errors as were found to be due to tryptophane. As the work progressed it soon became evident that the color due to tryptophane and its decomposition derivatives is quantitative for the amount of tryptophane present. The research was accordingly broadened so as to include the separate determination of both tyrosine and tryptophane.

Several years ago the observation was made in this laboratory (by T. Zucker) that cystine, though by itself substantially unaffected by either the phenol or the uric acid reagent, does give an intense blue color with the uric acid reagent—in the presence of sodium sulfite. The reaction is evidently due to the fact discovered by Heffter[7] that cystine is reduced to cysteine by the sulfite. The reaction with the uric acid reagent has not previously been developed into a quantitative method, but we have now used it for that purpose and believe that the process is worth incorporating in this paper as a quantitative colorimetric method for the determination of cystine.

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[6] Boil 15 gm. of molybdic oxide and 10 gm. of sodium hydroxide in 200 cc. of water until the solution no longer smells of ammonia. Then add 100 gm. of sodium tungstate, 50 cc. of 85 per cent phosphoric acid, 100 cc. of concentrated hydrochloric acid, and enough water to bring the volume up to 800 cc. Boil the mixture, for 10 hours, in a 1,500 cc. flask, fitted with a reflux condenser, and then remove the condenser and add a few drops of bromine to decolorize the solution. Boil off the excess of bromine, cool and filter the solution, and make up to a liter.

[7] Heffter, A., *Chem. Zentr.*, 1907, ii, 822.

In this paper are accordingly described colorimetric methods for the determination of (*a*) tyrosine, (*b*) tryptophane, and (*c*) cystine—in protein materials.

Separation and Estimation of Tyrosine and Tryptophane in Mixtures of Both.

As we happen to have on hand at the present time a considerable number of amino-acids in pure condition it was thought best to apply the phenol reaction to them although there was no reason to believe that any except tyrosine and tryptophane would give the reaction. No color was obtained with 10 mg. of each of the following amino-acids: arginine, lysine, aspartic acid, alanine, phenylalanine, histidine, glutamic acid, proline, leucine, valine, glycine, asparagine, cystine, isoleucine, isoserine. Of the seventeen amino-acids tested only tyrosine and tryptophane gave a color.

In this connection we would call attention to one erroneous statement found in the first paper on the uric acid and phenol reagents by Folin and Denis, an error emphasized by Gortner and Holm. Indole and its derivatives were said to give no reaction with the phenol reagent whereas in point of fact it does give an unmistakable though comparatively weak reaction. Indole gives no reaction with the uric acid reagent. The fact that the phenol reagent does give a reaction with indole has scarcely any bearing on the determination of tyrosine in proteins, though it is doubtless of some importance in other connections.

In order to determine the quantitative relationship in the color produced by tyrosine and by tryptophane the following experiments were made.

Experiment 1.—From stock solutions of each containing 1 mg. per cc., 2 mg. of the tryptophane and 1 mg. of tyrosine were transferred to separate 100 cc. volumetric flasks. 20 cc. of saturated sodium carbonate were added and enough water to give a volume of 50 cc. 2 cc. of the phenol reagent were added to each and after 30 minutes the flasks were filled to the mark and the color comparison was made. The tyrosine was used as a standard and was set at 20 mm. The tryptophane solution gave a reading 17.2 corresponding to 1.16 mg. of tyrosine. According to this result tryptophane gives 58 per cent of the color obtained from an equal weight of tyrosine.

Experiment 2.—100 mg. of tryptophane were boiled for 24 hours with 25 cc. of 20 per cent hydrochloric acid in a small Kjeldahl flask connected with a Hopkins condenser. The solution was cooled, diluted to 100 cc., and 2 cc., equivalent to 2 mg. of the tryptophane, were assayed as before, using 1 mg. of tyrosine as the standard. The color value was the same as with the original tryptophane. Boiling with acid does, therefore, not remove the disturbing effects of tryptophane in connection with tyrosine determinations.

From the preceding two experiments as well as from unrecorded repetitions of the same it is clear that in order to determine tyrosine in protein digestion mixtures containing tryptophane the latter must first be removed.

The quantitative, or substantially quantitative, removal of tryptophane from such digestion mixtures can be accomplished by means of the excellent mercuric sulfate reagent of Hopkins and Cole. But the usefulness of the process in connection with such small scale work as is involved in colorimetric analysis could be determined only by a systematic investigation. In addition to the question of how completely minute amounts of tyrosine and tryptophane could be separated it was recognized that the introduction of mercury carried with it the same sort of complications which were encountered from the silver salts in the analogous work on uric acid. It was found, however, that sodium cyanide is just as useful for the removal of mercury ions as for the removal of silver, and the necessary check work was thus much simplified.

Without going into a detailed description of the 26 different preliminary experiments which were made to determine the conditions for the quantitative separation of 1 mg. of tyrosine from 1 mg. of tryptophane the results may be summarized as follows: (a) If the acidity of the final solution represents less than 3.5 per cent of sulfuric acid there is danger of loss of tyrosine, due to its precipitation by (2 per cent) mercuric sulfate. (b) The tryptophane is quantitatively precipitated within 2 hours by 2 per cent mercuric sulfate, when the acidity lies between 3.5 and 7.5 per cent of sulfuric acid.

After having ascertained the conditions necessary for the separation and colorimetric estimation of tyrosine and tryptophane a series of analyses of various mixtures of the two was made in the following manner:

Standard solutions of tyrosine and tryptophane in 5 per cent sulfuric acid were prepared—each solution containing 1 mg. of amino-acid per cc.

By means of accurate 5 cc. burettes graduated in 0.02 of a cc. ("sugar burettes") definite amounts of the amino-acid solutions were measured into 15 cc. centrifuge tubes which had been graduated at a volume of 10 cc. 2 cc. of the mercuric sulfate solution (containing 10 per cent of mercuric sulfate and 5 per cent of sulfuric acid) were added and the mixture was at once diluted with 5 per cent sulfuric acid to the 10 cc. mark. A rubber stopper was inserted and the solution was shaken vigorously to insure thorough mixing. The tubes were· allowed to stand ,for 2 hours and were then centrifuged.

The clear supernatant liquid containing the tyrosine was poured into a clean dry test-tube and set aside for the tyrosine determination. The centrifuge tube containing the mercuric tryptophane sediment was then filled to the 10 cc. mark with 5 per cent sulfuric acid, its own rubber stopper was again inserted and the mixture was shaken. After removing the stopper and centrifuging, the supernatant liquid was carefully poured out and drained for half a minute.

For the subsequent colorimetric determinations of tyrosine and of tryptophane the process is as follows:

Tyrosine.—Transfer 5 cc. (one-half) of the tyrosine-containing liquid to a 100 cc. volumetric flask and into another similar flask introduce 1 cc. of the standard sulfuric acid-tyrosine solution containing 1 mg. of tyrosine. To the latter add also 1 cc. of the acid mercuric-sulfate solution and 3 cc. of 5 per cent sulfuric acid. Then add to each flask about 30 cc. of water, 20 cc. of saturated sodium carbonate solution, and 4 cc. of 5 per cent sodium cyanide solution, in the order named. It is best to shake for a moment after the addition of the carbonate, before adding the cyanide. The yellow mercuric carbonate thrown down by the sodium carbonate promptly dissolves on adding the cyanide and shaking. Add 2 cc. of the phenol reagent, mix, let stand for 10, or better for 30, minutes, and make the color comparison. in the usual manner, setting the standard at 20 mm.

20 times 2 divided by the reading of the unknown gives the tyrosine found, in milligrams.

Tryptophane.—The determination of the tryptophane in the mercury-tryptophane precipitate is not quite so simple as is the determination of the tyrosine in the supernatant solution. The precipitate dissolves readily and completely in an excess of sodium cyanide, but only under certain rather definite conditions. Moreover, the color obtained from 1 mg. of tryptophane in the form of the mercury compound is considerably deeper than the color obtained from a milligram of tryptophane not previously combined with mercury—even though the same amount of mercuric sulfate and sodium cyanide have been added before the addition of the phenol reagent. It is accordingly necessary to subject the tryptophane standard to the same treatment as the unknown; namely, to precipitate it with mercuric sulfate. This precipitation should, therefore, be started at the same time as the precipitation is made in the unknown or before. Since it makes no difference whether the precipitation is allowed to continue for 2 hours or for several days, a series of precipitated standards can, of course, be kept on hand in well stopped centrifuge tubes.

To the unknown mercury precipitate and to a similarly precipitated and centrifuged standard, containing 1 mg. of tryptophane, add 10 cc. of water, insert the rubber stopper, and shake so as to secure a uniform suspension. Without any unnecessary delay, that is within 2 or 3 minutes, add 4 cc. of 5 per cent sodium cyanide to each, insert the rubber stoppers, and mix. Complete solution occurs at once; whereas a fine, more or less gelatinous residue, containing tryptophane is left undissolved, if the preliminary shaking of the sediment is omitted.

Rinse the standard and the unknown into 100 cc. volumetric flasks, keeping the volumes approximately equal (50 cc.). Add first 20 cc. of sodium carbonate and finally, with shaking, 2 cc. of the phenol reagent. Let stand for not less than 10, preferably for 30, minutes, dilute to volume, and make the color comparison.

When the standard is set at 20 mm., 20 divided by the reading of the unknown in mm., gives, in milligrams, the amount of tryptophane present.

From the analytical figures recorded in Table I it will be seen that the process described above for the separate determination of tyrosine and tryptophane in various mixtures of the two yields substantially correct values.

TABLE I.

Illustrating Estimations of Tyrosine and Tryptophane in Mixtures.

Experiment No.	Amount taken.		Amount recovered.		Error.	
	Tyrosine.	Trypto-phane.	Tyrosine.	Trypto-phane.	Tyrosine.	Trypto-phane.
	mg.	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
1	1.80	5.00	1.82		+1.1	
2	1.60	0.00	1.60		0.0	
3	2.40	3.00	2.38		−0.9	
4	2.40	0.00	2.38		−0.9	
5	1.80	0.85	1.80	0.83	0.0	−2.3
6	2.20	1.20	2.18	1.16	−0.9	−3.3
7	2.10	0.90	2.08	0.89	−1.0	−1.0
8	2.00	1.00	1.94	0.98	−3.0	−2.0
9	1.90	0.95	1.90	0.93	0.0	−2.0
10	1.50	1.50	1.54	1.43	+2.6	−4.6
11	1.80	1.45	1.88	1.39	+4.4	−4.1
12	2.20	1.30	2.24	1.27	+1.8	−2.3
13	2.60	0.85	2.54	0.88	−2.3	+3.5
14	3.00	0.90	3.08	0.92	+3.6	+2.2
15	1.50	1.45	1.54	1.41	+2.6	−2.7
16	1.80	1.35	1.84	1.30	+2.2	−3.7
17	2.20	0.75	2.16	0.79	−1.8	+5.2
18	2.60	0.85	2.56	0.86	−1.5	+1.1
19	3.00	0.90	3.12	0.90	+4.0	0.0
20	0.00	1.30	0.00	1.29		−0.8
21	1.80	0.95		0.97		+2.0
22	0.00	0.80		0.81		+1.2
23	2.00	1.15		1.16		+0.9
24	0.00	1.20		1.18		+1.7

Colorimetric Determination of Cystine.

Since the colorimetric determination of cystine is based on the use of the uric acid reagent of Folin and Denis and since neither tryptophane, nor tyrosine, nor any other known amino-acid except cystine gives a reaction with this reagent the process for the determination of cystine in amino-acid mixtures is so simple as to require very little preliminary discussion.

As previously mentioned, Heffter found that cystine is reduced to cysteine by (10 per cent) sodium sulfite. Heffter made some use of this reaction in tests for cystine in tissue extracts by means

of nitroprusside; but, so far as we know, no attempt has been made to build up a quantitative method on the basis of these reactions; nor has the reducing effects of sulfites on cystine been worked out on a quantitative basis.

The figures recorded below (Table II) show that the quantitative or maximum conversion of cystine into cysteine is obtained only in the presence of a very large excess of sodium sulfite.

The experiments were made as follows: To 3 mg. of cystine in 100 cc volumetric flasks were added 20 cc. of saturated sodium carbonate and varying amounts of freshly prepared 20 per cent solution of sodium sulfite. After 5 minutes, 2 cc. of the uric acid reagent were added; the solutions were diluted to volume and the

TABLE II.

Showing the Amount of Sodium Sulfite Required for the Transformation of Cystine to Cysteine.

20 per cent Na$_2$SO$_3$ solution.	Colorimeter reading.	Amount of cystine found Theoretical value = 3 mg.
cc.	*mm.*	
1	39.0	1 53
2	32 2	1.86
3	29 3	2 05
4	28.5	2.10
5	25.8	2.31
10 (Standard)	20.0	3.00
20	20.0	3.00

color comparison was made, taking the one to which 10 cc. of sulfite had been added as the standard.

The amount of color obtained from different amounts of cystine is strictly proportionate to the quantities taken within the limits usually governing such color comparisons; that is, when neither the standard exceeds the unknown, nor the unknown the standard, by more than 50 per cent. In point of fact the proportionality is in this case dependable over still wider limits, but it is safer to keep within those mentioned.

The large amount of sulfite used for the reduction of the cystine has the same stabilizing effect on the color obtained as when sulfite is used with uric acid. This stabilizing effect is, however, not nearly so great as that obtained with sodium cyanide (and

uric acid). It is therefore essential that the color should be developed substantially simultaneously in the standard and the unknown. This rule is so frequently necessary in colorimetry that it may well be called one of its fundamental principles. If properly followed it makes no difference whether the color comparison, in cystine determinations, is made at the end of 10 minutes or after 1 hour, or even longer.

Sodium cyanide can scarcely be said to serve any useful purpose in connection with the colorimetric method for cystine in protein materials. If added *after* the blue color has once been developed it reduces somewhat the rate of the fading, but that fading is any way so slow as to render the addition of the cyanide quite superfluous. Moreover, if the cyanide is added in the usual way, that is *before* the introduction of the uric acid reagent it almost completely inhibits the development of the color. This inhibiting effect is obtained whether the cyanide is added before or after the addition of the sulfite.

Applications of the Colorimetric Methods for Tyrosine, Tryptophane, and Cystine to Protein Materials.

It would be highly desirable if tyrosine, tryptophane, and cystine could all be determined in a common hydrolysis mixture. It is doubtful, however, whether the most accurate results are obtainable under such conditions. For the cystine determination it is essential that the hydrolysis should be made with acid, since the cystine is completely destroyed by boiling with alkalies. On the other hand it seems to be generally agreed that the tryptophane survives better under alkali than under acid hydrolysis. The "humin" formation in acid hydrolysis, which Gortner and Holm have shown to involve more or less loss of tryptophane, constitutes an added reason against this form of hydrolysis for tryptophane determinations, although we are not yet quite sure to what extent tryptophane really loses its reactivity with the phenol reagent by virtue of such humin formation. Information on this point will doubtless come out of the promised further investigations by Gortner and Holm.

The tyrosine can be determined on the basis of either acid or alkali hydrolysis. For the determination of tyrosine and tryptophane we have used the following process:

The purified protein is dried for 48 hours in a vacuum desiccator over sulfuric acid.   1 gm. of the dried material is introduced into a 300 cc. long necked Kjeldahl flask.   After adding 3.5 gm. of crystallized barium hydroxide and 25 cc. of distilled water the neck of the flask is closed by a Hopkins condenser and the mixture is gently boiled over a micro burner for 40 to 48 hours.   30 cc. of 20 per cent sulfuric acid are then added and the flask is heated in boiling water for 30 to 60 minutes to drive off any hyrogen sulfide which may have formed.   The cooled mixture is transferred to a 100 cc. volumetric flask, diluted to the mark, thoroughly mixed, and filtered through a dry filter into a dry flask.   The amount of amino-acid-containing filtrate obtained is abundant for any reasonable number of tyrosine and tryptophane determinations, since 8 cc. of filtrate are about the maximum amount required for one pair of determinations.   The filtrate has also about the right degree of acidity for the separation of tyrosine and tryptophane.

Transfer from 1 to 8 cc. to a centrifuge tube graduated at 10 cc. Add 2 cc. of a solution containing 10 per cent of mercuric sulfate and 5 per cent of sulfuric acid (Hopkins and Cole reagent). Dilute with 5 per cent sulfuric acid to the 10 cc. mark.   Insert a rubber stopper and give a few vigorous shakes.   Let stand for 2 hours and then centrifuge.   The supernatant solution containing the tyrosine is decanted from the sediment which contains the tryptophane and these two fractions are then assayed colorimetrically as already described for pure mixtures of tryptophane and tyrosine (page 423).

The procedure described below has been found satisfactory for the determination of cystine:

From 1 to 5 gm. of the dry protein and 25 cc. of 20 per cent sulfuric acid are transferred to a 300 cc. Kjeldahl flask, fitted with a Hopkins condenser.   The mixture is boiled gently over a micro burner for 12 hours, after which it is cooled, diluted to 100 cc., and thoroughly mixed.   From 1 to 10 cc. of the solution are transferred to a 100 cc. volumetric flask and to it are added first 20 cc. of saturated sodium carbonate solution and then 10 cc. of 20 per cent sodium sulfite solution.   The mixture is well shaken and set aside while the standard cystine solutions are prepared.   The standard cystine solution is made to contain 5 per cent sulfuric acid and 1 mg. of cystine per cc.   This solution keeps indefinitely.

Two standards are prepared containing 1 and 3 cc., respectively, of cystine solution or 1 and 3 mg. of cystine. Add to each 20 cc. of saturated sodium carbonate solution and 10 cc. of 20 per cent sulfite and let stand for 5 minutes. 3 cc. of the uric acid reagent of Folin and Denis are then added (with shaking) to each standard and to the unknown digestion mixture. The three flasks are allowed to stand for 10 minutes; the contents are then diluted to the 100 cc. mark and the color comparison between the unknown and the standard nearest it in color is made in the usual manner. There is no need for any undue hurry in the making of the color comparison, for the slow fading which takes place is exactly the same in the unknown as in the case of the standard.

Amounts of Tyrosine, Tryptophane, and Cystine in Different Proteins.

We have purposely omitted in this paper to enter upon any discussions or criticisms of other methods previously described and used for the determination of tyrosine, tryptophane, and cystine in protein materials. In Tables III, IV, and V giving our analytical figures are also given corresponding figures taken from the literature and representing other methods.

Concerning these figures we would make the following brief comments:

1. Tyrosine.—Our colorimetric tyrosine figures are lower than the figures obtained by Folin and Denis. The difference is in the main due to the tryptophane though certain inconsistencies are evident—as in the case of edestin and gliadin. These inconsistencies are presumably due to different degrees of purity in the material used.

The main point about the tyrosine values, obtained by the colorimetric methods, is that they remain extraordinarily high in comparison with those obtained by gravimetric methods. Since "complete" analyses of protein materials always leave a very wide margin of unknown amino-acids we may for the present be permitted to assume that a part of unknown material consists of undetermined tyrosine.

2. Tryptophane.—In connection with the tryptophane values we would call attention to the last figure in Table IV showing that when 2 per cent of pure tryptophane was added to zein 2.01 per cent was found. Zein alone gave nothing.

TABLE III.

Tyrosine Content.

Protein.	Acid hydrolysis.	Ba(OH)₂ hydrolysis.	Folin and Denis.	Gravimetric.	Investigator.
	per cent	per cent	per cent	per cent	
Casein (Hammarsten)..........	5.36	5.32	6.5	4.5	Fischer, E., *Z. physiol. Chem.*, 1901, xxxiii, 151.
Wool..............	5.76	5.52	6.0	2.9	Abderhalden, E., and Voitinovici, A., *Z. physiol. Chem.*, 1907, lii, 349.
Zein (yellow corn).	5.6	5.52	5.5	3.6	Osborne, T. B., and Clapp, S. H., *Am. J. Physiol.*, 1907–08, xx, 477.
Horn..............		5.28	6.5	4.6	Fischer, E., and Dörpinghaus, T., *Z. physiol. Chem.*, 1902, xxxvi, 462.
Glutenin (wheat)..	4.7	4.5	5.8	4.25	Osborne, T. B., and Clapp, S. H., *Am. J. Physiol.*, 1906–07, xvii, 231.
Gliadin (wheat)....	3.5	3.4	3.3	2.4	Abderhalden, E., and Samuely, F., *Z. physiol. Chem.*, 1905, xliv, 276.
Serum globulin (cow)..........		6.7		2.5	Abderhalden, E., *Z. physiol. Chem.*, 1905, xliv, 17.
Ovalbumin........		4.2	5.0	1.77	Osborne, T. B., Jones, D. B., and Leavenworth, C. S., *Am. J. Physiol.*, 1909. xxiv, 252.
Witte peptone....	5.87	5.86		3.25	Levene, P. A., and Van Slyke, D. D., *Biochem. Z.*, 1908, xiii, 440.
Edestin (hemp seed)..........	5.70	5.76	5.2	2.1	Abderhalden, E., *Z. physiol. Chem.*, 1902–03, xxxvii, 499.
Fibrin.............	6.4	6.5		3.3	Levene, P. A., and Van Slyke, D. D., *Biochem. Z.*, 1908, xiii, 440.
Bence-Jones protein............		7.36		4.2	Hopkins, F. G., and Savory, H., *J. Physiol.*, 1911, xlii, 189.
Gelatin...........	Trace.	Trace.	Trace.	0	

TABLE IV.

Tryptophane Content.

Protein.	Au-thors.	Fürth[8] and Lieben.	Herz-feld.[9]	Fasal.[10]	Gravi-metric.	Investigator.
	per cent	per cent	per cent	per cent	per cent	
Casein (Hammar-sten)............	1.54	1.6	0.51	0.65	1.5	Hopkins, F. G., and Cole, S. W., *J. Physio'.*, 1901-02, xxvii, 418.
Wool..............	1.45		0.03			
Zein (yellow corn)..	0					
Horn.............	1.43			0.17		
Glutenin (wheat)..	1.68					
Gliadin (wheat)....	1.14				1.0	Abderhalden, E., and Samuely, F., *Z. physiol. Chem.*, 1905, xliv, 276.
Serum globulin (cow)............	2.28	4.4				
Ovalbumin.	1.23	2.6	0.52			
Witte peptone	3.03	5.3	1.25			
Edestin (hemp seed)............	1.40	3.0	0.58	0.38		
Fibrin.............	2.90	5.3	1.05		1.3	Neuberg, C., and Popowsky, N., *Biochem. Z.*, 1907, ii, 357.
Bence-Jones pro-tein..............	1.67					Hopkins, F. G., and Savory, H., *J. Physiol.*, 1911, xlii, 189.
Gelatin............	0					
Zein plus 2 per cent tryptophane.....	2.01					

In the case of tryptophane it is to be noted that others have attempted to determine it by colorimetric methods, the latest work being that of Fürth and Noble.[11] The figures which these

[8] Fürth, O., and Lieben, F., *Biochem. Z.*, 1920, cix, 124.

[9] Herzfeld, E., *Biochem. Z.*, 1913, lvi, 258.

[10] Fasal, H., *Biochem. Z.*, 1912, xliv, 392.

[11] Fürth, O., and Nobel, E., *Biochem. Z.*, 1920, cix, 103.

authors have obtained are so very much higher than those given by our method that either their procedure or ours manifestly must be fallacious. Fürth and Noble admit errors amounting to 27 per cent when known amounts of pure tryptophane are added to gelatin. We do not think that any experienced investigator could obtain anything approaching such errors by our method.

TABLE V.

Cystine Content.

Protein.	Colori-metric.	Gravi-metric.	Investigator
	per cent	*per cent*	
Horn....................	6.67	6.8	Mörner, K. A. H., *Z. physiol. Chem.*, 1901–02, xxxiv, 207.
Wool....................	7.8	7.3	Abderhalden, E., and Voitinovici, A., *Z. physiol. Chem.*, 1907, lii, 348.
Fibrin....................	3.5	1.2	Mörner, K. A. H., *Z. physiol. Chem.*, 1901–02, xxxiv, 207.
Glutenin (wheat)........	1.80	0.02	Osborne, T. B., and Clapp, S. H., *Am. J. Physiol.*, 1906–07, xvii, 231.
Gliadin (wheat).........	2.32	0.5	" " "
Zein (yellow corn)......	0.5		
Casein (Hammarsten)...	0.25	0.1	Mörner, K. A. H., *Z. physiol. Chem.*, 1901–02, xxxiv, 207.
Edestin..................	0.75	0.3	Abderhalden, E., *Z. physiol. Chem.*, 1902–03, xxxvii, 499.
Gelatin..................	0.2		
Witte peptone...........	2.00		
Human hair.............	16.50	14.0	Buchtala, H., *Z. physiol. Chem.*, 1907, lii, 474.

3. *Cystine.*—It is interesting to note that the gravimetric determination of cystine in the keratins, which are rich in cystine, has been accomplished with quite a fair degree of accuracy. The cystine figures recorded for other proteins, as might have been expected, are much less dependable.

FREE AND BOUND WATER IN THE BLOOD.

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To explain certain pathologic conditions and even normal functions of the body, assumptions have been made that the body water is partly bound and partly free. From a chemical standpoint the body water could be bound in two ways—by hydration of the ions and by imbibition by proteins.

Many facts from electrical endosmosis, transference experiments, and the relation between conductivity and viscosity, all point to hydration of the ions. When, however, one begins to determine the relative hydration of the different ions, the results obtained differ with the method used. Thus Washburn and his coworkers (1) from transference and viscosity data assign to H ion, K ion, and Cl ion hydration values of 0, 1.3, and 5.4 molecules of H_2O, respectively. On the other hand, Bjerrum (2) on the basis of the lowering of the freezing point and electromotive force measurements, calculates the respective values for these ions as 8 H_2O for H ion, 2 H_2O for Cl ion, and 0 H_2O for K ion. On the basis of the theory of von Hevesy (3) one would expect maximum hydration for the H ion. The effects of pressure on the degree of ionization and the phenomena of electrostriction lead to the idea that very probably all the solvent molecules are attracted by electrostatic forces to the ions, the attractive force being some function of the distance. This view has been recently advanced by Akerlof (4) to explain his remarkable results on the effect of salts on the activity of H ion as well as by Keller (5) in his discussion of the significance of the isoelectric point. While conceivably the attraction between the solvent molecules and the ions might vary, any appreciable change in the ratio of bound to free water would not be caused, as the body water has but a low salt concentration.

The great factor, however, in binding water would be the imbibition by the colloids. It is well known that lyophilic colloids such as the blood proteins will imbibe large quantities of water, and that as the quantity imbibed increases, the pressure necessary to cause partial desorption becomes less. Highly dispersed particles, which present adsorbing surfaces, are subject to great compressing forces, as has been shown, especially by Williams (6). A change in the quantity or character of the surface active constituents in the blood would entail a change in the adsorption by the colloids and a consequent change in the compressing force upon them. Since the proteins constitute about 8 per cent of the blood serum, a marked change in the quantity of water imbibed would be of import and on such a basis a theory of bound and free water could be founded. It has, however, been found by Hofmeister (7) and others that in the case of the swelling of gelatin in salt solutions the concentration of these salts in the imbibed water and in the solution is practically equal. Should this likewise be the case with the body proteins, the quantity of water imbibed would have no effect on the colligative properties of blood serum; as a consequence, bound or free water could not be detected by the use of these properties.

Burgarszky and Tangl (8) determined the freezing point lowering of dog's serum and found values between -0.550 to -0.639, corresponding to concentrations of 0.297 to 0.354 molar. An average of 55 determinations by different investigators is given by Hamburger (9) as -0.571 corresponding to 0.308 mols.

To note how near these values, approached those at $37\frac{1}{2}$°C., determinations of the lowering of the vapor pressure of blood serum at about $37\frac{1}{2}$°C., compared to that of pure water at the same temperature, were, therefore, made. Alveolar air was bubbled through the serum, and air through the pure water, and then in each case through sulfuric acid. For the same volume of air passed through, the difference in the quantity of water absorbed by the sulfuric acid through which the air from the pure water passed, and that absorbed by the sulfuric acid through which the alveolar air from the serum passed was proportional to the difference in vapor pressure. This value divided by the weight absorbed by the sulfuric acid through which the air from the pure water passed gave the relative lowering in vapor pressure.

The value of this obtained as an average of six experiments, in which no value differed by more than 5 per cent from the mean was $\frac{0.09}{13}$. If serum be considered a dilute solution, the following relation holds:

$$\frac{dP}{P} = \frac{n}{N}$$

In this relation dP equals the difference in vapor pressure between the pure solvent and solution; P the vapor pressure of the solvent; n the number of mols of solute; and N the number of mols of solvent. If we substitute for $\frac{dP}{P}$ its value $\frac{0.09}{13}$, and for N the number of mols of water in 1,000 gm., *i.e.* 55.55 mols, we obtain for "n" a value of 0.384 mols.

There are two complete analyses of dog's serum given by Abderhalden (10). If the averages of the two values given are recalculated in molecules per 1,000 gm. of water, and following Burgarszky and Tangl's assumptions that the NaCl is about 84 per cent dissociated and that the balance of Na, K, and other cations may be considered as equivalent to an equal concentration of Na_2CO_3 with a degree of dissociation of about 69 per cent, one obtains a molar or (as Hamburger has termed it) "osmotic concentration" of 0.3298 mols per 1,000 gm. of water. Considering the unavoidable errors in a dynamic determination of lowering of vapor pressure the agreement is good.[1]

The fair agreement, moreover, between the calculated molar concentration from the analyses, from the lowering in freezing point, and from the lowering in vapor pressure, supports the conclusion that water imbibed by proteins, as in the case of gelatin, carries practically the same concentration of salts, so that any theory of bound and free water cannot be confirmed or disproved by any physicochemical method based on colligative properties.

The author desires to thank Professor E. K. Marshall, Jr. for the samples of blood serum.

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[1] It should be borne in mind that Abderhalden's results do not contain any figures for CO_2, likewise the values from the freezing point determinations are probably too low because of the loss of some of the CO_2.

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THE PROTEINS OF THE TOMATO SEED, SOLANUM ESCULENTUM.*

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According to Rabak (1), the total quantity of tomato seed waste which accumulates annually in the United States as a by-product of tomato pulping plants varies with the pack of the particular season and with the variety of tomato used. From figures determined by several experiments, American grown tomatoes contain on an average 1.13 per cent of dry waste of which 46.1 per cent is seeds. Shrader and Rabak (2) found "that a total of 2,063 tons of seed is available as the output of all the pulping plants in the eastern and middle western tomato belts." Most of this seed has gone to waste, some being dumped into rivers and some allowed to rot near the packing plants. A little has been tried as fertilizer.

The seed and press-cake used for the experiments described in this paper were obtained from different sources. Total nitrogen determinations were made, the results of which are given in Table I.

The meal used in preparing the proteins was obtained by grinding seeds of high germinating quality and removing most of the oil by extracting with U.S.P. ether. Thus prepared the meal contained an average of 37.28 per cent of protein (N × 6.25).

Two globulins, designated α and β, have been isolated from the saline extracts and separated by fractional precipitation with ammonium sulfate. According to the elementary analyses,

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* A preliminary report of this paper was presented at the 15th annual meeting of the Society of Biological Chemists held in Chicago, December 28 to 30, 1920 (*cf.* Johns, C. O., and Gersdorff, C. E. F., *J. Biol. Chem.* 1921, xlvi, p. xxvi).

TABLE I.

Analyses of Seed and Press-Cake.

Description.	Total N.	Protein (N × 6.25).
	per cent	*per cent*
Press-cake* (protein coagulated by heat of pressing)..	5.78	36.13
" † (seeds heated to 60°C. before pressing)....	5.90	36.88
Seeds‡ (unheated, oil extracted by ether).............	5.96	37.23
	5.83	36.42
Seeds§ (highest germinating quality, oil extracted by ether)...	6.08	37.91
Average...	5.91	36.91

* This material was furnished by the Oil, Fat and Wax Laboratory, Bureau of Chemistry, U. S. Department of Agriculture, Washington.

† This material was furnished by the Bureau of Plant Industry, U. S. Department of Agriculture, Washington.

‡ These seeds were obtained in the open market.

§ These seeds were furnished by the Office of Congressional Seed Distribution, U. S. Department of Agriculture, Washington.

these globulins exhibit marked differences in certain of their constituents. The average composition of the two globulins, calculated on a moisture- and ash-free basis, is as follows:

.	C	H	N	S	O
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
α-globulin................	52.29	6.89	18.34	1.16	21.32
β- "	51.21	6.84	16.02	0.81	25.12

It has been found that the α-globulin coagulates on heating for 10 minutes at 74°C., while the β-globulin in the same period coagulates at 96°C. Both globulins give qualitative tests for tryptophane and tyrosine.

In the distribution of the basic amino-acids as determined by the Van Slyke method, a marked difference is shown in the content of histidine, the α-globulin being low in this amino-acid, while it is unusually high in the β compound. The content of arginine and lysine in both globulins is high. In the distribution of the nitrogen calculated on the basis of the weight of the protein, from the analyses made by the Van Slyke method, the greatest difference appears to be in the content of the non-basic nitrogen.

Feeding experiments on the nutritive value of the seeds after the removal of the oil, which proved to be of excellent table quality (3), have been made by Finks and Johns (4), who found that the proteins were nutritionally adequate, normal growth of albino rats being obtained. Our chemical study confirms these conclusions.

Tests failed to disclose the presence of albumin and glutelin.

EXPERIMENTAL.

Experiments on Press-Cake.

Our first experiments were made on a press-cake which contained 36.13 per cent of protein (N × 6.25). It was found that the heat developed during the pressing had rendered the proteins insoluble in the ordinary saline solvents. A number of preparations of protein were made, however, by extracting the finely ground press-cake with 0.5 per cent solution of sodium hydroxide. These preparations were uniformly alike in composition, but differed from the preparations obtained from the saline extracts of the ether-extracted meal by having much lower sulfur and nitrogen contents and higher carbon. These lower sulfur and nitrogen results may have been due to the action of the alkali used as the solvent, and the higher content of carbon to the admixture of carbohydrates. These preparations were a light buff in color.

Total Globulins Extracted by 0.5 Per Cent Sodium Hydroxide Solution.—Extractions were made, using 10 cc. of solvent to each gram of meal. The quantity of meal used for a preparation ranged from 25 to 500 gm. Extractions ranged from 48 to 96 hours. Duration of the extractions with these time limits had no appreciable effect on yield or analysis, 23.63 per cent of protein being extracted (N × 6.25), corresponding to a yield of 65.40 per cent based on the total protein in the meal.

The clear extracts obtained after being filtered through paper pulp were acidified with 1 per cent acetic acid, the acid being added slowly until the flocculent precipitates coagulated sufficiently to settle. The precipitates were collected on folded filter paper and washed several times with distilled water. They were then redissolved in a 0.2 per cent sodium hydroxide solution,

filtered, precipitated as before, and washed with distilled water until the washings gave no tests for acidity. They were then washed with absolute alcohol four times and once with absolute ether and dried in a vacuum oven at 110°C. The yield averaged 11.2 per cent. The preparations were then exposed to the air for 72 hours to enable them to acquire a moisture equilibrium, so as to facilitate the weighing of samples for analysis.

The average analyses of five preparations thus obtained showed the following composition, calculated on a moisture- and ash-free basis:

C	H	N	S
per cent	per cent	per cent	per cent
54.12	6.88	15.06	0.84

Our subsequent experiments were made on seeds specially selected for their high germinating quality, this being an assurance that they had never been subjected to a temperature that would coagulate the proteins.

Experiments on Seeds.

Preliminary Experiments.—Extraction experiments were made with different concentrations of sodium chloride in water, extracting for 1 hour in each experiment. Nitrogen[1] determinations were made on aliquot portions of the filtered extracts and percentages of protein extracted calculated from these results. The data are given in Table II. Time extraction experiments were next made with the solvent which extracted the maximum amount of protein. These experiments show that a 4 per cent sodium chloride solution, in 1 hour, extracts the maximum amount of protein—21.34 per cent (N × 6.25). These results are given in Table III.

Test for Glutelin.—Exhaustive extraction experiments were made using a 4 per cent sodium chloride solution. Three extractions were made with this solvent, at the rate of 8 cc. for the

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first and third, and 10 cc. for the second, for each gram of meal, extracting for periods of 1 hour each. The extracts were filtered and measured. Nitrogen determinations were made on aliquot portions of the extracts measured, allowance being made in case of the third extract for the residual extract in the meal. After the third extract had been removed, the residue was washed once with sodium chloride solution and then several times with dis-

TABLE II.

Preliminary Extraction Experiments.

Solvent: 8 cc. per gram of meal, extracted 1 hour at room temperature.

Solvent.	N extracted.	Protein extracted (N × 6.25).
	per cent	*per cent*
Distilled water............................	0.80	5.02
Sodium chloride:		
1.0 per cent............................	1.63	10.18
2.0 " "	2.02	12.64
3.0 " "	2.86	17.90
4.0 " "	3.42	21.34
4.5 " "	3.35	20.92
5.0 " "	3.15	19.66
5.5 " "	3.01	18.81
6.0 " "	2.76	17.27
10.0 " "	2.72	16.99

TABLE III.

Time Extraction Experiments.

Solvent: 4 per cent sodium chloride solution, 8 cc. per gram of meal.

Time extracted, *hours*............................	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	3
Protein extracted (N × 6.25), *per cent**..	19.59	21.34	21.06	18.81	18.88

* Based on the total volume of solvent used.

tilled water. A fourth extraction was then made, using a solution of 0.5 per cent sodium hydroxide at the rate of 10 cc. of solvent per gram of meal, extracting for a period of 2 days. As shown by a nitrogen determination on the filtered alkali extract, no protein was extracted by this solvent. The tomato seed, therefore, does not contain a glutelin. The results of these experiments are given in Table IV.

Test for Albumin.—Extractions were made, using 7 cc. of distilled water per gram of meal, extracting for periods of 1 hour each. The filtered extracts were dialyzed against chilled running water for 17 days. The dialysates were filtered clear, acidified for 3 hours with washed carbon dioxide to precipitate traces of globulins, and again filtered. Samples tested according to the procedure of Johns and Waterman (5), after slight acidification with a 1 per cent acetic acid solution, failed to give a coagulum even after

TABLE IV.

Test for Glutelin.

Exhaustive Extraction Experiments.

Solvent.	Total N extracted.	Protein extracted (N × 6.25).
	per cent	*per cent*
4 per cent sodium chloride, first extraction, 8 cc. per gram of meal. Extracted 1 hour....................	2.38	14.88*
4 per cent sodium chloride, second extraction, 10 cc. per gram of meal. Extracted 1 hour....................	1.38	8.63*
4 per cent sodium chloride, third extraction, 8 cc. per gram of meal. Extracted 1 hour....................	0.36	2.25†
0.5 per cent sodium hydroxide, fourth extraction, 10 cc. per gram of meal. Extracted 2 days..............	0.00	0.00
Total extracted....................................	4.12	25.76‡
" in meal.......................................	5.96	37.23
Not accounted for	1.84	11.47

* Protein extracted was based on volume of extract obtained.

† Protein extracted was based on volume of extract obtained plus residual extract in meal.

‡ Protein extracted, based on total protein in meal, was 69.19 per cent.

extended boiling, indicating the absence of an albumin in the tomato seed.

Globulins Extracted by Sodium Chloride Solution and Fractionally Precipitated by Ammonium Sulfate.—Extractions were made using 8 cc. of 4 per cent sodium chloride solution per gram of meal.   From 150 to 1,000 gm. of meal were used for each preparation.   The meal was mixed with the solvent, all lumps being disintegrated by hand, and the mixtures were allowed to stand at room temperature for 1 hour, with occasional stirring.   The

mixtures were then poured onto large Buchner funnels fitted with two circular pieces of cheese-cloth, and filtered by suction, the residue being washed twice with 4 per cent sodium chloride solution. The extracts so obtained were filtered clear through mats of filter paper pulp on Buchner funnels. The extracts were then fractionated with ammonium sulfate.

Preparation of the α-Globulin.—The clearly filtered extracts were measured, and made 0.3 saturated with solid ammonium sulfate, allowing the mixtures to stand over night. The precipitates were collected on folded filter paper and washed with solutions of the same salts and concentration as those from which they were precipitated in order to remove traces of the other fractions which might have been adsorbed. The precipitated protein was then suspended in 4 per cent sodium chloride solutions (Preparations I, and III to VII), dissolved in a large volume of aqueous 4 per cent sodium chloride (Preparation II), and dialyzed against chilled running water for 10 days. The preparations were then separated from the dialysates, washed free of chlorides and sulfates, and dried and allowed to come to a moisture equilibrium in the air, as described in the preparation of the mixed globulins. The average yield was 4.12 per cent.

Because of the marked tendency of the α-globulin to denature, the difficulty with which it redissolved made it necessary to dialyze this protein in suspension.

One preparation of α-globulin was obtained from a dialysate (Preparation VIII), after removal of the precipitated protein, by coagulation following acidification, by boiling the solution for 5 to 10 minutes. This was washed and dried in the same manner as the other preparations. The yield was 0.72 per cent.

In elementary analysis preparations of the denatured and coagulated proteins of the α-globulin resembled closely the soluble portion. Analyses of eight preparations, calculated on a moisture- and ash-free basis, are given in Table V.

Preparation of the β-Globulin.—The filtrates from the α preparations were made 0.4 saturated with ammonium sulfate, and the precipitates thus obtained were removed by filtration and discarded. The clear filtrates were then made saturated, precipitating the β-globulin. These fractions were easily dissolved in small quantities of distilled water, dialyzed, washed, dried, and

treated as previously described.   The average yield was 0.57 per
cent.

When precipitated from saline solutions the quantity of β-
globulin appeared to be so much greater than that actually
obtained on dialysis, that investigations were made on the dialy-
sates from these preparations.   Further dialysis against chilled
distilled water failed to precipitate more protein.   On saturation
with washed carbon dioxide after the second dialysis, however, a
precipitate (Preparation III, Table VI) was obtained—a yield of
additional protein of 0.10 per cent.   The gummy nature of this
fraction rendered its separation from the mother liquor by filtra-
tion very difficult.   On making the filtered dialysates slightly
acid with a 1 per cent acetic acid solution and then heating the
solutions for 10 to 20 minutes at 96°C., coagulated protein precip-
itates were obtained in average yields of 0.78 per cent (Prepara-
tions IV and V, Table VI).   These preparations, after thorough
washing with distilled water, were dried and treated as before
described.   Analyses of Preparations III, IV, and V showed them
to be identical in composition with Preparations I and II of the
β-globulin.   The results of these analyses calculated on a mois-
ture- and ash-free basis are given in Table VI.

Physical Properties of the Globulins.—The two globulins were in
finely powdered form, the α ranging in color from white to creamy
white and the β from pale buff to buff.

Tests of the solubility of the globulins disclosed the fact that
the α-globulin is easily denatured.   In order to redissolve this
protein it is necessary that it be handled promptly, requiring very
large volumes of 4 per cent sodium chloride solution.   The
β-globulin, on the other hand, is easily soluble in a solution con-
taining only minute traces of sodium chloride, which property
interferes with the complete precipitation of all of this globulin
by dialysis.

Tests to determine the coagulation points of these globulins
were made on redissolved portions of the fractionated proteins
in 4 per cent sodium chloride solution, after precipitation by
dialysis.   Starting with clear solutions made slightly acid with
1 per cent acetic acid, the proteins were coagulated by the pro-
cedure of Johns and Waterman (5).   The α-globulin coagulated in
10 minutes at 74°C. and the β-globulin in the same time at 96°C.

The precipitation limits of the two globulins were clearly defined. Between the 0.3 and 0.4, and the 0.4 and 0.5 points of

TABLE V.

*Analyses of the α-Globulin.**

	Preparation I.			Preparation II.			Preparation III.		
.	I	II	Average.	I	II	Average.	I	II	Average.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
C............	52.77	52.52	52.65	52.50	52.50	52.50	52.51	52.34	52.43
H............	6.94	6.99	6.97	6.86	6.79	6.83	6.96	6.81	6.89
N............	18.28	18.42	18.35	18.50	18.57	18.54	18.24	18.44	18.34
S............		1.16	1.16		1.17	1.17		1.06	1.06
O............			20.87			20.96			21.28
Moisture.....		5.42			7.14			6.32	
Ash.........		0.29			0.02			0.18	
	Preparation IV.			Preparation V.			Preparation VI		
C............	52.18	52.23	52.21	51.82	51.92	51.87	52.47	52.51	52.49
H............	6.78	6.96	6.87	6.84	6.83	6.84	6.86	7.11	6.99
N............	18.21	18.18	18.20	18 38	18.27	18.33	18.63	18.48	18.56
S............		1.16	1.16		1.18	1.18		1.15	1.15
O............			21.56			21.78			20.81
Moisture.....		5.61			6.85			5.50	
Ash.........		0.17			0.05			0.28	
	Preparation VII.			Preparation VIII.			Average of eight preparations.		
C............	51.78	51.97	51.88	52.30	52.31	52.31		52.29	
H............	6.84	6.90	6.87	6.94	6.74	6.84		6.89	
N............	18.26	18.10	18.18	18.27	18.23	18.25		18.34	
S............		1.10	1.10		1.28	1.28		1.16	
O............			21.97			21.32		21.32	
Moisture.....		5.43			5.83				
Ash.........		0.39			0.16				

* Calculated on moisture- and ash-free basis.

saturation with ammonium sulfate after the removal of the middle fraction at 0.4 of saturation, there is practically no separation of protein. The middle fraction with a sulfur content of 0.89

per cent is evidently a mixture containing merely traces of the α-globulin.

Qualitative Tests.—By the Hopkins and Cole method, the α-globulin gave a strong positive test for tryptophane, the color ring developing immediately. The β-globulin, however, gave but a slight test, a faintly colored ring developing in about 10 minutes.

TABLE VI.

Analyses of the β-Globulin. *

	Preparation I.			Preparation II.			Preparation III.		
	I	II	Average.	I	II	Average.	I	II	Average.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
C............	51.43	51.48	51.46	51.07	51.16	51.12	51.25	51.02	51.14
H............	6.79	6.86	6.83	6.77	6.98	6.88	6.71	6.72	6.72
N............	16.02	15.97	16.00	16.03	16.04	16.04	16.01	16.05	16.03
S............		0.80	0.80	0.80	0.79	0.80		0.79	0.79
O............			24.91			25.16			25.32
Moisture.....		6.59			7.16			9.28	
Ash.........		1.99			1.62			0.13	

	Preparation IV.			Preparation V.			Average of five preparations.		
C............	51.21	51.07	51.14	51.21	51.19	51.20	51.21		
H........:...	6.87	6.83	6.85	6.92	6.96	6.94	6.84		
N............	16.04	16.08	16.06	15.97	15.96	15.97	16.02		
S............		0.82	0.82		0.82	0.82	0.81		
O............			25.13			25.06	25.12		
Moisture.....		6.41			6.28				
Ash.........		0.30			0.29				

* Calculated on moisture- and ash-free basis.

By Millon's test, the presence of tyrosine in both globulins was disclosed.

Distribution of the Nitrogen in the α- and β-Globulins as Determined by the Van Slyke Method.—Duplicate samples weighing 3.0 gm. each were dissolved in 100 cc. of 20 per cent hydrochloric acid and hydrolyzed by boiling the solutions under condensers for 30 hours. The phosphotungstates of the bases were decom-

posed by the amyl alcohol-ether method (6).   The results of
these analyses are given in Tables VII, VIII, IX, X, and XI.   The

TABLE VII.

*Distribution of Nitrogen in the α-Globulin as Determined by the Van Slyke
Method.**

Sample I.   Ash- and moisture-free, 2.7852 gm. protein, 0.5164 gm.
nitrogen.†

Sample II.   Ash- and moisture-free, 2.7852 gm. protein, 0.5164 gm.
nitrogen.†

	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N...........................	0.0597	0.0601	11.56	11.64	11.60
Humin N adsorbed by lime..............	0.0033	0.0032	0.64	0.62	0.63
" N in ether-amyl alcohol extract..	0.0080	0.0083	1.55	1.61	1.58
Cystine N...........................	0.0041	0.0042	0.79	0.81	0.80
Arginine N..........................	0.1253	0.1250	24.27	24.21	24.24
Histidine N.........................	0.0077	0.0098	1.49	1.90	1.70
Lysine N............................	0.0267	0.0255	5.17	4.94	5.05
Amino N of filtrate...................	0.2772	0.2756	53.68	53.37	53.52
Non-amino N of filtrate...............	0.0087	0.0091	1.69	1.76	1.73
Total N regained....................	0.5207	0.5208	100.84	100.86	100.85

* Total N regained corrected for solubility of the bases (7).
† Nitrogen content of protein, 18.54 per cent.

TABLE VIII.

Basic Amino-Acids in α-Globulin.

	I	II	Average.
	per cent	per cent	per cent
Cystine.........................	1.26	1.29	1.28
Arginine.........................	13.98	13.95	13.97
Histidine........:.................	1.02	1.30	1.16
Lysine..........................	5.00	4.78	4.89
Tryptophane.....................			Present.

figures for cystine are undoubtedly low, since this amino-acid is
slowly decomposed in boiling hydrochloric acid.

TABLE IX.

Distribution of Nitrogen in the β-Globulin as Determined by the Van Slyke Method.*

Sample I. Ash- and moisture-free, 2.7366 gm. protein, 0.4389 gm. nitrogen.†

Sample II. Ash- and moisture-free, 2.7366 gm. protein, 0.4389 gm. nitrogen.†

	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N	0.0510	0.0516	11.62	11.75	11.69
Humin N adsorbed by lime	0.0071	0.0073	1.62	1.66	1.64
" N in ether-amyl alcohol extract	0.0013	0.0012	0.30	0.27	0.28
Cystine N	0.0036	0.0037	0.82	0.84	0.83
Arginine N	0.0941	0.0934	21.44	21.28	21.36
Histidine N	0.0265	0.0299	6.04	6.81	6.43
Lysine N	0.0340	0.0326	7.75	7.43	7.59
Amino N of filtrate	0.2052	0.2028	46.75	46.20	46.47
Non-amino N of filtrate	0.0206	0.0185	4.69	4.22	4.46
Total N regained	0.4434	0.4410	101.03	100.46	100.75

* Total N regained corrected for solubility of the bases (7).

† Nitrogen content of protein, 16.04 per cent.

TABLE X.

Basic Amino-Acids in β-Globulin.

	I	II	Average.
	per cent	per cent	per cent
Cystine	1.13	1.16	1.14
Arginine	10.69	10.61	10.65
Histidine	3.57	4.03	3.80
Lysine	6.48	6.21	6.35
Tryptophane			Present.

TABLE XI.

Distribution of Nitrogen in the α- and β-Globulins as Calculated from the Van Slyke Analyses in Terms of Per Cent of the Proteins.

N	α-Globulin.			β-Globulin.		
	I	II	Average.	I	II	Average.
	per cent	per cent	per cent	per cent	per cent	per cent
Amide	2.14	2.16	2.15	1.86	1.89	1.87
Humin	0.41	0.41	0.41	0.31	0.31	0.31
Basic	5.88	5.91	5.90	5.78	5.83	5.81
Non-basic	10.26	10.22	10.24	8.25	8.09	8.17
Total	18.69	18.70	18.70	16.20	16.12	16.16

SUMMARY.

Analyses of five samples of tomato seed and press-cake show an average content of 36.91 per cent of protein (N × 6.25). The total globulins were extracted from the press-cake by extraction with 0.5 per cent sodium hydroxide. Preparations obtained from these extractions gave uniform results on analysis. From seed of high germinating quality two globulins, α and β, have been isolated and analyzed. These globulins, coagulable by heating for 10 minutes at 74 and 96°C., are respectively precipitated from their saline solutions by 0.3 of saturation in the case of the α-globulin, and by saturation with ammonium sulfate in the case of the β-globulin. They differ in their solubility, the α-globulin being easily denatured, while the β compound is highly soluble in mere traces of aqueous sodium chloride. The elementary analyses of the two globulins show the following percentage differences in composition:

	C	N	S
	per cent	*per cent*	*per cent*
α-globulin.................	52 29	18.34	1.16
β- "	51.21	16 02	0.81

The sulfur content of the globulins is in the ratio of 3:2.

The analyses by the Van Slyke method show that the nutritionally essential basic amino-acids are well represented in these globulins. It is of interest to note that these globulins are high both in arginine and lysine. The β-globulin contains also an unusually large percentage of histidine, but in the α-globulin the histidine content is low. Both globulins respond to the qualitative tests for tryptophane and tyrosine. In the distribution of the nitrogen based on the weight of the proteins, as calculated from the Van Slyke analyses, the most marked difference is found in the figures for the non-basic nitrogen.

Tests failed to disclose the presence of albumin and glutelin.

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A COLORIMETRIC METHOD FOR THE DETERMINA-
TION OF HOMOGENTISIC ACID IN URINE.

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School of Medicine, St. Louis.)*

When *o*-phosphates are added to ammonium molybdate in acid solution, the well known ammonium phosphomolybdate is formed, which corresponds to the 1:24 type of phosphomolybdic acid described by Wu.[1] Bell and Doisy[2] have found this complex to be especially susceptible to reduction by hydroquinone; a deep green coloration is produced in acid solution which is changed to an intense blue when made alkaline; the excess ammonium molybdate is not reduced. On a basis of those reactions they have devised methods for the colorimetric determination of phosphorus in blood and urine.

While carrying out the tests for homogentisic acid in an alcapton urine the probability arose that this *p*-diphenol would give the same color with phosphomolybdic acid as hydroquinone, and such was found to be the case. Subsequently the homogentisic acid was isolated, its color ratio to hydroquinone determined, and then hydroquinone used as the standard in the quantitative determination of homogentisic acid.

The method is very simple: 1 or 2 cc. of the alcapton urine are diluted to about 15 cc. in a 25 cc. volumetric flask, 2 cc. of the molybdate solution and 2 cc. of the phosphate solution are added, and the mixture is diluted with water to the mark. Into another flask containing equal amounts of the phosphate and molybdate solutions an appropriate amount of hydroquinone standard is added and diluted with water up to the mark. The flasks are inverted a few times so that the contents are well mixed, and the comparison is made in the colorimeter after 5 minutes.

[1] Wu, H., *J. Biol. Chem.*, 1920, xliii, 189.
[2] Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, 1920, xliv, 55.

Solutions Used.

1. Phosphate solution contains 1 per cent KH_2PO_4.
2. Molybdate solution contains 5 per cent ammonium molybdate in 5 N H_2SO_4.
3. Hydroquinone standard contains 1 mg. of hydroquinone per cc.

The average of several comparisons gave 1 mg. of hydroquinone equal to 0.79 mg. of homogentisic acid. It will be observed that this is not a molecular ratio.

Various substances which might be expected to interfere with the colorimetric method, such as uric acid and phenols, give relatively little color. This was determined by adding to a series of flasks, each containing 1 mg. of homogentisic acid, 1 mg. of the substance to be tested. Phosphate and molybdate solutions were then added as in the determination and the colors obtained compared with that of 1 mg. of homogentisic acid alone. The following substances gave an error of about 1 per cent: phenol, cresol, resorcinol, and uric acid; pyrocatechol freed from traces of hydroquinone gave an error of about 3 per cent. Sulfides give a very intense blue-green; if present, however, they can be removed by precipitation with Ag_2SO_4. Albumin, if present, would interfere by forming a cloud; it may be removed by trichloroacetic acid. Sulfides and albumin may be conveniently removed together by treating the urine with equal volumes of CCl_3COOH (10 per cent) and Ag_2SO_4 (0.5 per cent); this mixture is stirred well, solid NaCl to make about 2 per cent is added, the mixture stirred again, and then centrifuged. 3 cc. of the supernatant fluid are taken for 1 cc. of urine in the determination.

Determinations of homogentisic acid made by the colorimetric method and by that of Wolkow and Baumann[1] gave results which checked within about 10 per cent. The method of Wolkow and Baumann is described as very tedious by all who have used it, so the colorimetric method offers a more convenient means for studying the metabolic anomaly of alcaptonuria.

[1] Wolkow, M., and Baumann, E., Z. *physiol. Chem.*, 1891, xv, 260.

THE RELATION OF PHOTOSYNTHESIS TO THE PRODUCTION OF VITAMINE A IN PLANTS.*

By J. WALTER WILSON.

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That the synthesis of vitamine A takes place only in plants is generally believed. The green parts of plants appear to be among the richest sources of the vitamine, whereas seeds, in general, contain only traces of it (1–5). It seems apparent, therefore, that the plant must synthesize the vitamine A found so abundantly in its leaves. Since some plant syntheses are dependent upon solar energy it appeared desirable to find out whether or not the production of vitamine A is dependent upon photosynthesis. Recently Coward and Drummond (5) concluded that the amount of vitamine A does not increase in germination and that it is only increased in those plant tissues in which photosynthesis is going on. The following experiments have led me to a different conclusion; *viz.*, that photosynthesis is not necessary for the production of vitamine A in plants.

EXPERIMENTAL.

Wheat seeds were sprouted in wooden trays on moist paper, one lot in the dark room and another in the sunlight. When the sprouts were from 2 to 3 inches high, they were cut off close to the seeds, dried in a current of air at about 60°C. (Osborne and Mendel, 2), and ground into fine powders. These were fed to young white rats in a diet deficient in vitamine A, 5 per cent of the powdered sprouts taking the place of an equivalent weight of corn-starch (Chart 1).

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* From a thesis presented in partial fulfillment of requirements for the degree of Doctor of Philosophy, Brown University, 1921.
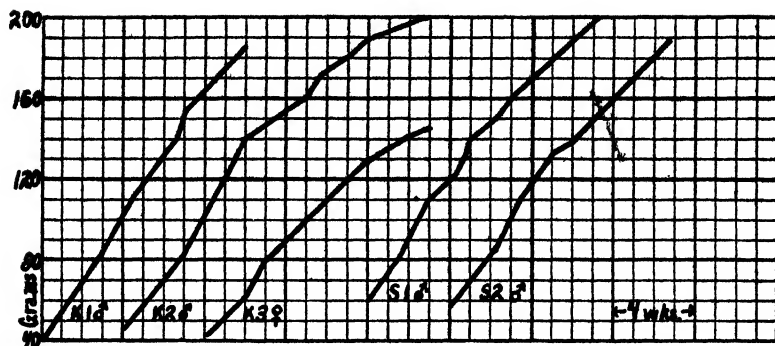
CHART 1. Showing that photosynthesis is not necessary for the production of vitamine A in plants.

Rats K1, K2, and K3 were fed the basal diet with 5 per cent of dried etiolated wheat sprouts replacing an equivalent amount of corn-starch.

Rats S1 and S2 were fed the basal diet with 5 per cent of dried green wheat sprouts replacing an equivalent amount of corn-starch.

The basal diet was made up as follows:

	gm.
Casein	20
Corn-starch	50
Lard	20
Salt mixture	5
Yeast	5

The salt mixture was that of McCollum and Davis (6). The casein was prepared from a commercial product[1] by washing it in a shaking machine with 2.5 per cent acetic acid, 50 per cent alcohol, 95 per cent alcohol, and ether, successively. It was shaken at least 2 hours with an abundance of each fluid and allowed to stand sometime before being filtered. It was finally spread in thin layers and dried. The yeast used was a dried commercial product sold as "Fleischmann's Autolyzed."[2] That the basal diet is adequate for the growth of white rats in all respects excepting the vitamine A content is shown in Chart 2.

[1] Obtained from the Casein Manufacturing Co., Bainbridge, N. Y., as D₁ Casein.

[2] Obtained from Arthur H. Thomas Co., Philadelphia, Pa.
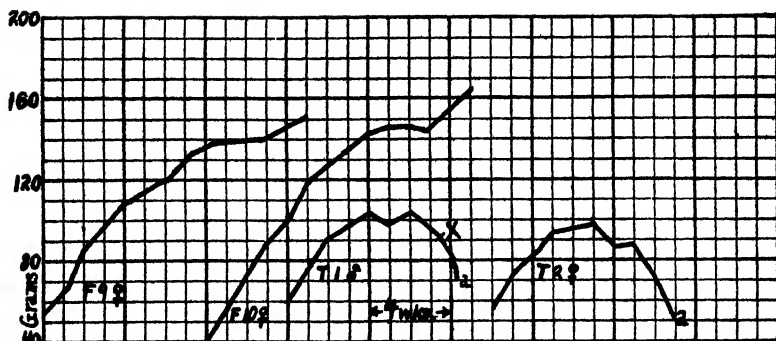
CHART 2. Showing that the basal diet used in these experiments is adequate, except that it lacks vitamine A.

Rats T1 and T2 were fed the basal diet (p. 456). Rat T1 developed keratomalacia at the point marked X.

Rats F9 and F10 were fed the same diet except that 10 per cent of butter fat was substituted for an equivalent amount of lard.
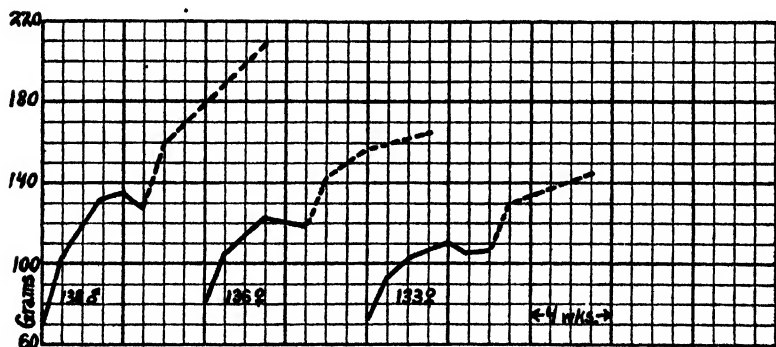
CHART 3. Showing the recovery of rats beginning to decline on a diet deficient in vitamine A when dried etiolated wheat sprouts were added to the diet.

During the period indicated by the solid line these rats were fed on the basal diet.

During the period indicated by the broken line 8 per cent of the dried etiolated wheat sprouts were substituted for an equivalent amount of corn-starch in the diet.

The rats used in these experiments were obtained from a stock bred in the laboratory especially for this work. They are kept in wooden cages with galvanized iron pans containing a sawdust litter. The stock rats are fed on a biscuit prepared by Potter and Wrightington of Boston (Ferry, 7). This is supplemented occasionally by fresh carrots; and pregnant and lactating females receive in addition a mash of fresh milk and stale bread. All the rats are watered by the inverted bottle method. The dates of birth of all litters are recorded and only such animals as have shown normal growth up to the time of experiment have been used.

DISCUSSION.

The growth curves obtained (Charts 1 and 3) show that wheat sprouts, grown either in the dark or in the light, are adequate sources of vitamine A when they make up 5 per cent of the diet. The 5 gm. of dried etiolated sprouts in 100 gm. of the diet represented not more than 20 gm. of whole wheat seeds, as is shown by the following figures:

Weights of 2 lots of 25 seeds .... 0.363 gm. and 0.367 gm.
  "   " 2 "   " 25 dried etiolated sprouts. 0 098 "   "   0.107 "

Inasmuch as McCollum and Davis (8) have shown that even 64 per cent of wheat in the diet does not carry an adequate amount of vitamine A, it is evident that this factor must have increased in the etiolated as well as in the green sprouts. It seems improbable, however, that the increase would be equal in both sorts of sprouts, because all physiological activity must be more intense in the sprouts grown in the light than in those grown in the dark. Indeed, it is possible that Coward and Drummond (5) have merely shown in their experiments that the increase in vitamine A is more rapid in green than in etiolated sprouts; and that the quantity of sprouts fed by them was insufficient to detect the smaller production in the latter. It may be remarked, however, that in the table of their paper,[3] most of the etiolated sprouts fed are shown to be "slightly active" although the amounts used were considerably smaller than in my experiments.

SUMMARY.

Either etiolated or green wheat sprouts furnish an adequate amount of vitamine A when the dried sprouts make up 5 per cent

[3] Coward and Drummond (5), p. 538.

of the diet of white rats. Inasmuch as this proportion of sprouts represents a quantity of seeds which, if included in the diet, would be inadequate as a source of this vitamine, the conclusion is drawn that vitamine A is produced in the growing plant with or without any accompanying photosynthesis.

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THE INTERACTION BETWEEN BLOOD SERUM AND TISSUE EXTRACT IN THE COAGULATION OF THE BLOOD.

I. THE COMBINED ACTION OF SERUM AND TISSUE EXTRACT ON FLUORIDE, HIRUDIN, AND PEPTONE PLASMA; THE EFFECT OF HEATING ON THE SERUM.

By LEO LOEB, MOYER S. FLEISHER, and LUCIUS TUTTLE.

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On former occasions we have discussed repeatedly the action of tissue extract and serum separately, as well as their combined action on fluoride, hirudin, and peptone plasma of vertebrate blood.[1] We pointed out the loss in power of a mixture of serum and tissue extract on standing and the difference in the behavior of fluoride plasma (and similar plasmas) in which coagulation had been prevented through inactivation of calcium on the one hand, and of hirudin and peptone plasma on the other hand. We also determined how far the specific adaptation of the tissue coagulins depended upon a combination with a constituent of the blood serum and on the direct action of these substances on the coagulable plasma. In this and the following paper we shall report on a continuation of these experiments and especially shall we analyze the changes which take place in a mixture of tissue extract and blood serum on standing.[2] A discussion of our con-

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[1] Loeb, L., *Montreal Med. J.*, 1903, xxxii, 507; *Med. News.*, 1903, lxxxiii, 212; *Beitr. chem. Physiol. u. Path.*, 1904, v, 534; 1907, ix, 201; *Virchows Arch. path. Anat.*, 1904, clxxvi, 10; *Biochem. Centr.*, 1907, vi, 829, 889; *Z. Immunitätsforsch., Orig.*, 1912, xii, 189. Loeb, L., and Fleisher, M. S., *Biochem. Z.*, 1910, xxviii, 169.

[2] These experiments were carried out more than 12 years ago. We published some of our results in a short note. In particular did we mention the effect of moderate heat on tissue extract (Loeb, L., *Z. Immunitätsforsch., Orig.*, 1912, xii, 189). A number of questions raised in this paper needed

clusions shall be given in the second part of our paper. We shall use in these papers the following abbreviations: D = dog; R = rabbit; C = cat; Gp = guinea pig; G = goose; FlPl = fluoride plasma; HPl = hirudin plasma; PPl = peptone plasma; TE = tissue extract; KE = kidney extract; ME = muscle extract; LE = liver extract; SplE = spleen extract; Cp = stroma of erythrocytes; S = serum.

The Combined Action of Serum and Tissue Extract on Fluoride Plasma.

Fluoride plasma in the case of the dog and rabbit was obtained by receiving the blood in an equal amount of a 6 to 7 per cent NaFl solution and centrifuging; in the case of the goose a 5 per cent solution was used. The mixture of serum and extract is added at once to the fluoride plasma. If we combine various kinds of extracts and sera in definite proportions (in a large number of experiments 0.3 cc. of tissue extract was mixed with 0.7 cc. of serum, and the quantity of plasma used was 1 cc.), and add at once the mixture of serum and extract to the plasma, the results vary somewhat in different cases, indicating the presence of variable factors. We find that such a combination causes an acceleration of the coagulation which is more considerable than would correspond to a mere summation of the effects of extract and serum in a large number of cases, and much more frequently than if the same combination were used with peptone and hirudin plasma. The result varies, however, with combinations· of extract and serum from different species, but even with the same kind of combinations variations occur with the extracts and sera from different individuals. Thus with a combination of DKE+DS in many cases an activation occurs, but not always; when GS takes the place of DS as the serum component in the mixture a lack of acceleration is more frequent than with DS. The effect of a combination of extract and serum on fluoride plasma is not merely determined by the relation of serum

further experimental analysis. We had hoped to be able to complete these experiments and delayed, therefore, the full publication of our investigation. At present, however, we are still unable to carry our work further and we thought it, therefore, best to report on the results obtained so far.

and extract to each other, but even more so by the relation of each serum and extract individually to the plasma. If both alone are active on a plasma, they are so in combination. If the extract is inactive, it may even inhibit an otherwise active serum. The specific relation of extract to the plasma may be apparent even in fluoride plasma, whenever the extracts alone are strong enough to cause coagulation; and this specific relation may sometimes be apparent even in combination with serum; but in this case again the specificity of the extract generally refers to the plasma and not the serum. Thus GME tends to act better with GFlPl than with DFlPl independently of the kind of serum admixed to the extract. However, in some cases an isocombination of serum and extract $(DS + DE, GS + GE)$ seems to act better independently of the plasma to which it is added. On the whole, activation caused by the mixture of serum and extract is much clearer if DFlPl and RFlPl are used than with GFlPl.

If the mixture is allowed to stand for a short time at room temperature before being added to the fluoride plasma, a noticeable decrease in its coagulating effect is generally observed, but this loss is on the whole much smaller than in the case of hirudin or peptone plasma.

The intensity of the loss and the character of the curve which represents the loss as a function of the time during which extract and serum had a chance to act upon each other before being added to the plasma depend upon the combination of serum and extract used. With DS+DKE the loss is usually greatest; with GS+GME it is variable, sometimes it is considerable, at other times it is smaller, quite generally it is less than with DS+DKE. With a combination of GS+DKE the decrease in activity on standing is not so great as with DS+DKE, and with DS + GME the loss is usually the least; there may be even a gain after standing 6 minutes in the case of the latter combination. The combinations RS + DKE and RS + GME showed usually little loss. In addition there may be some difference in the effect of a mixture with different kinds of plasma; GS+GME, for instance, may act differently with DFlPl and with GFlPl.

If we arrange the mixtures of various kinds of sera and extracts in a definite order in accordance with their behavior

towards DFlPl we usually find the order to be very similar if DHPl is used instead of DFlPl, and even with the hirudin and fluoride plasma of the rabbit and goose the order indicating the loss of coagulating power of certain combinations on standing remains similar.   Certain deviations may, however, occur.

The time curve varies with different combinations of serum and extract.   If we use a combination of DS+DKE with DFlPl there is in typical cases an optimum after 30 seconds to 1 or 2 minutes.   The coagulation time of a mixture which has been standing ½ to 2 minutes before being added to the FlPl is often shorter than that of a mixture added at once.   If the mixture stands longer a progressive prolongation of the coagulation time occurs with the time of standing.   This deterioration varies, it may be less than proportional to time, proportional to time, or more than proportional to time; but it is always considerably less than with hirudin plasma.   There may at first be a loss which is less than proportional to time and later it may become more than proportional to time.   This curve could be explained by assuming that two processes go hand in hand, namely the production of an accelerating as well as of an inhibiting substance, and that the former may in the first period of standing of the mixture predominate over the latter.   The fact that with fluoride plasma the loss of the mixture in coagulating power after standing is much less than if we use hirudin plasma, can be understood if we assume that in the case of FlPl the serum is the important factor in the mixture, while with hirudin and peptone plasma, on the contrary, it is the extract; and that as a result of standing the coagulating factor in the serum is less affected than the coagulating factor in the extract.

During the optimal period (½ to 1 minute) after the mixing, the coagulation time of the FlPl may be 50 per cent shorter than if the mixture had been added to the plasma at once.   With a combination of DLE and DS a similar optimum was found, but it happened to be a little later than at 1 minute and deterioration likewise set in somewhat later; namely 3 minutes after the mixing.   With GS+GME no definite optimum was observed, but during the first few minutes following the mixing of serum and extract the coagulation time remained about the same; this was followed by a deterioration.   The absolute amount of loss

following the optimal period varies in different cases. In a DS+DKE mixture the loss varies on the average between 200 and 600 per cent after 6 minutes standing. In extreme cases it may amount to as much as 2,000 per cent, or it may be as little as 50 to 100 per cent. With the other combinations of serum and extract the loss in time is usually correspondingly less in the order indicated above (the loss being smallest with DS+GME). GS+GME may lose 400 per cent in the first 3 minutes and no further change may be noticeable after 6 minutes. In an experiment in which with DS+DKE the loss was 400 to 500 per cent after 12 minutes, it was 300 per cent with GS+GME. In a case in which DS+DKE lost 400 per cent after 6 minutes, GS+DKE lost only 250 per cent. DS+GME or RS+GME may even show a gain after 6 minutes standing.

The curve of deterioration is also influenced by the proportion of serum contained in the mixture. With more serum the loss seems to be greater; but this relation comes out much more clearly with HPl than with FlPl. With FlPl some irregularities occurred in this connection. In a case in which extract had lost in power through standing 24 hours before being used, and had become less active in consequence, the time curve of loss in combination with serum remained almost the same.

As we have seen there is often an optimum between 30 seconds and 1 minute if DS and DKE are mixed. In several experiments it was found, however, that the coagulation of FlPl could be still more accelerated if serum was first added to the FlPl and the extract immediately afterwards.

Action of Serum and Tissue Extract on Fluoride Plasma.

Time of standing of DS + DKE.	Mixture.	Coagulation time.	
min.		min.	sec.
0	1 cc. DFlPl + 0.7 cc. DS + 0.5 cc. DKE	25	
5	1 " " + 0.7 " " + 0.5 " "	3	27
10	1 " " ' + 0.7 " " + 0.5 " "	4	35
		Mostly coagulated; remains incomplete.	

Time of standing of RS + DKE.	Mixture.	Coagulation time.
min.		sec.
0	1 cc. DFlPl + 0.7 cc. RS + 0.5 cc. DKE	40
5	1 " " + 0.7 " " + 0.5 " "	38
10	1 " " + 0.7 " " + 0.5 " "	56

Time of standing of DS + DKE.	Mixture.	Coagulation time.	
min.		min.	sec.
0	1 cc. DFlPl + 0.5 cc. DKE + 1.2 cc. 0.85 per cent NaCl	1	
1	1 cc. DFlPl + 0.5 cc. DKE + 0.5 cc. DS		43
2	1 " " + 0.5 " " + 0.5 " "		59
4	1 " " + 0.5 " " + 0.5 " "	1	55
8	1 " " + 0.5 " " + 0.5 " "	2	49
12	1 " " + 0.5 " " + 0.5 " "	3	28

Time of standing of DS + DKE.	Mixture.	Coagulation time.	
min.		min	sec
0	1 cc. DFlPl + 0.7 cc. DS + 0.3 cc. DKE	4	35
½	1 " " + 0.7 " " + 0.3 " "	3	5
1	1 " " + 0.7 " " + 0.3 " "	3	40
2	1 " " + 0.7 " " + 0.3 " "	4	
3	1 " " + 0.7 " " + 0.3 " "	5	35
6	1 " " + 0.7 " " + 0.3 " "	9	30
10	1 " " + 0.7 " " + 0.3 " "	16	45

Time of standing of DS + DLE.	Mixture.	Coagulation time.	
min.		min	sec.
0	1 cc. DFlPl + 0.7 cc. DS + 0.3 cc. DLE	4	40
½	1 " " + 0.7 " " + 0.3 " "	3	55
1	1 " " + 0.7 " " + 0.3 " "	3	30
2	1 " " + 0.7 " " + 0.3 " "	4	5
3	1 " " + 0.7 " " + 0.3 " "	4	20
6	1 " " + 0.7 " " + 0.3 " "	6	5
10	1 " " + 0.7 " " + 0.3 " "	7	50

Mixture.	Coagulation time.
	min. sec.
1 cc. DFlPl + 0.7 cc. DS	22 10
1 " " + 1 " DKE	47 40
1 " " + 1 " DLE	Next morning, trace of coagulation.

Time of standing of S + E.	Mixture.			Coagulation time.	
min.				*min.*	*sec.*
0	1 cc. DFlPl + 0.7 cc. DS + 0.3 cc. DKE			1	20
6	1 " " + 0.7 " " + 0.3 " "			3	10
0	1 " " + 0.7 " " + 0.3 " GME			4	50
6	1 " " + 0.7 " " + 0.3 " "			3	50
0	1 " " + 0.7 " RS 0.3 " DKE			1	10
6	1 " " + 0.7 " " + 0.3 " "			1	25
0	1 " " + 0.7 " GS + 0.3 " "			2	5
6	1 " " + 0.7 " " + 0.3 " "			3	20
0	1 " " + 0.7 " " + 0.3 " GME			5	30
6	1 " " + 0.7 " " + 0.3 " "			17	15

The Combined Action of Serum and Tissue Extract on Hirudin and Peptone Plasma.

Our hirudin plasma was prepared by adding 12 to 18 mg. of hirudin to 100 cc. of dog or rabbit blood, and 5 mg. of hirudin to 100 cc. of goose blood. As in the case of the fluoride plasma we have to add less anticoagulating substance to bird than to mammalian blood in order to obtain a relatively stable and spontaneously non-coagulable (during the period of the experiment) plasma. This fact accords with the experience that bird blood is naturally much more stable than mammalian blood.

The mixture of serum and extract is at once added to the hirudin plasma. With DHPl a mixture of extract and serum varies in its effect from slight inhibition to slight acceleration, as compared with the effect of extract alone on the plasma. As to the effects of various combinations great variations are found. With DHPl,

DS+DKE is often inhibiting, but it may not be inhibiting or it may even be slightly accelerating. Also GS+GME may be inhibiting. GS+DKE may be inhibiting, but cross-mixtures are perhaps more often less inhibiting than isocombinations like DS+DKE or GS+GME; this, however, does not always hold good. Thus with GHPl, DS and RS may inhibit an active GME, while GS may either inhibit it, or may be indifferent or even slightly accelerated. As in the case of FlPl, RS was on the whole found less inhibiting than the other sera. Isocombinations do not show any special activation. Dog or rabbit tissue extract which alone is ineffective with GHPl can usually not be made effective through combination with a serum of the same species. On the whole, if a particular kind of serum or extract alone acts well with hirudin plasma, it acts also best in combinations. Thus GS may vary in combination as it does alone, and at one time it may slightly accelerate one kind of extract, in another case another extract. In case GS alone is less effective with GHPl, it is apt to inhibit also GME to a greater extent than other sera. In the same way in different cases, DKE may be either inhibited or slightly accelerated with DS or GS; if the mixture is added to GHPl in the same experiment, GS may inhibit GME with GHPl and it may accelerate DKE and RKE with the same plasma. These variations make it probable that in addition to the differences in the strength of an extract characteristic of a species there are differences in the strength of individual extracts of a species.

The activation called forth through a combination of serum and extract is on the average much less noticeable with hirudin plasma than with fluoride plasma.

If the mixture of serum and extract is allowed to stand for some time at room temperature, before being added to the plasma, the loss on standing is in general much greater with hirudin plasma than with fluoride plasma; but again the character of the time curves varies with the combination used.

With a mixture of DS+DKE there may be slight inhibition as compared to DKE alone if the mixture is added at once to the hirudin plasma; at other times there may be slight acceleration. There is usually no gain after a short time of standing but a continuous loss; under certain conditions there may be a slight

transitory gain.   At first the loss may be less than proportional to time, then it becomes proportional to time, and soon it increases to a point where coagulation is indefinitely delayed.   The steepness of the curve increases, therefore, rapidly.   The turning point varies in different experiments, but it lies between 3 and 8 minutes in the majority of the experiments.   After standing for 8 to 12 minutes the coagulation may be prevented altogether.

DS inhibits equally with DKE and RKE and DSplE.   With DLE the inhibition seems to be less and the same seems to hold good, perhaps even to a higher degree, with RLE.   This applies at least to the LE which we used in our experiments.   Thus there was in a mixture of DS+DLE a slight inhibition, if the mixture was added at once.   After 30 seconds standing there was a slight improvement and this was followed by a steady decrease which was, however, less than proportional to time.   Again two processes go apparently hand in hand, an accelerating and an inhibiting one.   The larger the amount of the inhibiting substance in the serum, the more the inhibition prevails.

A combination of GS+GME shows variable results.   There may be a steadily increasing loss up to 6 minutes when the experiment was suspended; or as in the case of DS+DKE there may be a turning point after 3 minutes standing.   In certain cross-mixtures the loss is less marked.   GS inhibits usually with DKE much less than does DS; but it varies somewhat in individual cases.   There may be some inhibition at once; this may be followed by a steadily but slowly increasing loss, amounting to about 110 per cent after 12 minutes standing.   Or the main loss may occur in the first 3 to 4 minutes; this loss may be slight (80 to 100 per cent) or it may be greater.   This is followed by a period in which there is no further loss or only a very slight loss. The mixture GS+RKE may lose much on standing.   Again with DS+GME there may be hardly any loss on standing.

With RS in combination with DKE or GME the loss on standing was usually much less than with DS; but the actual loss depends upon the amount of RS added and on the time of standing. With 0.7 cc. of RS in combination with 0.3 cc. of DKE there may be no inhibition after 10 minutes standing, but the inhibition may become quite marked if we increase the quantity of RS and the time of standing.   With 1.2 cc. of RS there may be

no loss after 6 minutes standing; but after 12 minutes the inhibition may be considerable.   With 1.4 cc. of RS the inhibition may be very marked as early as after 6 minutes standing, or even earlier, the inhibition increasing more and more with the longer standing of the mixture.

If instead of using DHPl we use GHPl the mixtures behave similarly.   Certain differences, however, exist.   GME gains relatively in efficiency with GHPl as compared with mammalian extracts and, therefore, mixtures of GS+GME are relatively active; while combinations of DKE with DS or with GS are relatively inactive.   But here too DS+DKE or DS+RKE lose usually much on standing, while DS+GME lose little or may even gain, and the loss of GS+DKE is generally less than that of DS+DKE.   GS+GME shows a variable loss.   In these experiments with GHPl, RS usually had a markedly inhibiting effect on extracts.   With RHPl the mixture of extracts and sera behaved about as it did with DHPl.   A mixture of DS+RKE showed much loss on standing.   RS+GME was not so favorable as DS+GME.   RS+RKE usually was much inhibited.

On the whole if we have to deal with a very active extract or serum, which alone are very active with hirudin plasma, the inhibition of this mixture on standing may become as slight with HPl as with FlPl; but usually it is much greater with HPl than with FlPl.· There is, however, an unmistakable parallelism in the effect of the various kinds of mixtures on fluoride plasma and hirudin plasma.

Effect of Variations in the Amount of Serum on the Activity of the Mixture.

If we add the mixture of serum and extract to the hirudin plasma at once, the serum may cause a slight inhibition of the extract, or it may prove indifferent, or it even may cause a slight acceleration.   But if we increase the amount of serum in the mixture added to the plasma at once, the inhibition becomes distinct when it had not been apparent with a smaller amount of serum.   Thus variability in the effect of the mixture may depend on differences in the amount of inhibiting substance present in a special serum and in the quantity of the serum added.

An increase in the amount of serum causes likewise an increase in the inhibition of the mixture after standing. The curves representing the increase in inhibition as a function of the quantity of serum vary, however, in accordance with the kind of serum and extract used. The inhibition increases most with an increase in the quantity of serum in the combination DS+DKE. The quantity curve of the mixture taken after a constant time of standing of the mixture shows the same steepness and sharp turning point which we find in the curve representing the increase in inhibition as a function of time of standing of the mixture with a constant quantity of serum. In both cases there is a very steep increase in the coagulation time, which soon reaches the point where coagulation is delayed indefinitely. The sharp turning point in combination with 0.3 cc. of DKE, and after standing of the mixture for 4 minutes, may be as low as somewhere between 0.2 and 0.4 cc. of DS, or it may be between 0.6 and 0.8 cc. of DS. The coagulation time increases more than proportionally to the amount of DS added.

In the mixture GS+GME an increase in the amount of GS likewise increases the coagulation time, but much less steeply than in the case of DS+DKE.

We already stated that an increase of RS (from 0.7 to 1.4 cc.) caused a marked increase in inhibition, when with smaller quantities the inhibiting effect of RS after standing was not apparent, or when it was even slightly accelerating under those conditions.

An increase in the amount of extract in the mixture has the opposite effect; it increases the rapidity with which the mixture causes coagulation of the hirudin plasma and diminishes the inhibition with serum. In a mixture in which an addition of extract may prevent inhibition, provided the mixture is added at once to the hirudin plasma, it may again become very pronounced after 12 minutes standing of the mixture so that coagulation is delayed indefinitely. During the period of standing the inhibiting substance of the serum has been able to combine with the extract in such a way that the coagulating effect of the mixture has been destroyed. The turning point in time is just as sharp with a somewhat larger quantity of extract as with a smaller quantity.

From the character of the time curves it follows that at a given time the inhibition observed in a mixture of serum and extract may vary greatly in different cases. Thus in DS+DKE the loss after 6 to 10 minutes standing may vary between 400 and 3,000 per cent, or in other cases no coagulation may take place. After 10 to 12 minutes standing the loss may vary between 2,500 per cent and infinity. But it may happen in a special case that the loss of the mixtures DS+DKE after standing 12 minutes amounts to only 150 per cent. GS+GME usually shows a correspondingly somewhat smaller loss; it may amount to 700 to 1,400 per cent with DHPl and 100 per cent with GHPl after 6 minutes standing.

In the cross-mixtures the loss was usually much less. In a mixture of GS+DKE after 6 minutes standing it varied between 50 and 700 per cent. In an experiment in which with this mixture the loss was 700 per cent, it amounted to 100 per cent with DS+GE, in the latter mixture the loss being smallest.

In general the results are very similar, if we use peptone plasma of the dog instead of hirudin plasma. Our experiments with peptone plasma are, however, less numerous than those with hirudin plasma.

The Action of Serum and Extract on Hirudin and Peptone Plasma.

· Mixture.	Coagulation time.
	min.　sec.
1 cc. DHPl + 0.5　cc. DKE	1　28
1 "　　" 　+ 0.25 " 　"	2　32
1 "　　"　 + 2　　" DS	Coagulated next morning.
1 "　　"　 + 2　　" GS	"　　　"　　　"
1 "　　"　 + 1　　" GKE	Not yet coagulated next morning.
1 "　　"　 + 0.5 "　　"	"　　"　　　"　　　"　　　"

Time of standing of S + E.	Mixture.	Coagulation time.
min.		min. sec.
0	1 cc. DHPl + 0.5 cc. DKE + 0.7 cc. DS	3 50
4	1 " " + 0.5 " " + 0.7 " "	Coagulated next morning.
6	1 " " + 0.5 " " + 0.7 " "	Almost coagulated next morning.
12	1 " " + 0.5 " " + 0.7 " "	No coagulation next morning.
0	1 " " + 0.5 " " + 0.7 " GS	6 43
4	1 " " + 0.5 " " + 0.7 " "	14 30
6	1 " " + 0.5 " " + 0.7 " "	13 58
12	1 " " + 0.5 " " + 0.7 " "	13 30
0	1 " " + 0.3 " " + 0.7 " "	21 5
1	1 " " + 0.3 " " + 0.7 " "	28 46
4	1 " " + 0.3 " " + 0.7 " "	36 30
6	1 " " + 0.3 " " + 0.7 " "	33 10
12	1 " " + 0.3 " " + 0.7 " "	43 Incomplete. 83 Complete coagulation.
1	1 " " + 0.5 " GME + 0.7 " "	Trace after 2 hrs.

Effect of quantity of serum. S + E were allowed to stand for 4 minutes before being added to the HPl.

Mixture.	Coagulation time.
	min. sec.
1 cc. DHPl + 0.5 cc. DKE + 0.2 cc. DS	8
1 " " + 0.5 " " + 0.4 " "	A great part coagulated after 2½ hrs.
1 " " + 0.5 " " + 0.6 " "	
1 " " + 0.5 " " + 0.8 " "	A smaller part coagulated after 2½ hrs.
1 " " + 0.5 " " + 0.2 " GS	5 28
1 " " + 0.5 " " + 0.4 " "	13 5
1 " " + 0.5 " " + 0.6 " "	13 10
1 " " + 0.5 " " + 0.8 " "	18 8
1 " " + 0.5 " " + 1.2 " "	30
1 " " + 0.3 " " + 0.7 " 0.85 per cent NaCl	7
1 cc. DHPl + 0.5 cc. DKE + 0.5 cc. 0.85 per cent NaCl	6 30
1 cc. DHPl + 0.7 cc. DKE + 0.3 cc. 0.85 per cent NaCl	5 25

Time of stand-ing of S + E.	Mixture.		Coagulation time.	
min.			*min.*	*sec.*
0	1 cc. DHPl + 0.7 cc. DS + 0.3 cc. DKE		7	
½	1 " " + 0.7 " " + 0.3 " "		11	50
2	1 " " + 0.7 " " + 0.3 " "		14	35
3	1 " " + 0.7 " " + 0.3 " "		10	45 Mostly. Next morning coagulated.
6	1 " " + 0.7 " " + 0.3 " "		14	10 Partly. Next morning partly coagulated.
10	1 " " + 0.7 " " + 0.3 " "		"	" "

Mixture.		Coagulation time.	
		min.	*sec.*
1 cc. DHPl + 0.7 cc. DS + 0.3 cc. DLE		5	35
1 " " + 0.7 " " + 0.3 " "		5	10
1 " " + 0.7 " " + 0.3 " "		6	50
1 " " + 0.7 " " + 0.3 " "		8	45
1 " " + 0.7 " " + 0.3 " "		11	35
1 " " + 0.7 " " + 0.3 " "		16	10

Time of standing of S + E.	Mixture.			Coagulation time.		
min.				hrs.	min.	sec.
	1 cc. DPPl + 1 cc. DKE				2	20
	1 " " + 0.5 " "				1	45
	1 " " + 0.3 " "				3	10
	1 " " + 2 " DS			Next morning partly coagulated.		
	1 " " + 1 " "			Next morning not coagulated.		
0	1 " " + 0.7 " " + 0.3 cc. DKE				9	45
2	1 " " + 0.7 " " + 0.3 " "			1	8	Trace.
				Next morning almost coagulated.		
6	1 " " + 0.7 " " + 0.3 " "			Next morning trace.		
10	1 " " + 0.7 " " + 0.3 " "			Next morning trace.		
0	1 " " + 0.5 " " + 0.5 " "				2	45
2	1 " " + 0.5 " " + 0.5 " "				23	Partly coagulated.
				Next morning almost coagulated.		
0	1 cc. DHPl + 0.7 " " + 3 " "				3	55
6	1 " " + 0.7 " " + 3 " "			4	7	30
0	1 " " + 0.7 " " + 0.3 " GME			Mostly 30 min., incomplete next morning.		
6	1 " " + 0.7 " " + 0.3 " "			3	2	40
0	1 " " + 0.7 " RS + 0.3 " DKE				1	30
6	1 " " + 0.7 " " + 0.3 " "				4	10
0	1 " . " + 0.7 " GS + 0.3 " "				3	50
6	1 " " + 0.7 " " + 0.3 " "				4	15
0	1 " " + 0.7 " " + 0.3 " GME			2	23	Partly.
				Next morning almost coagulated.		
6	1 " " + 0.7 " " + 0.3 " "			3	2	Half coagulated.
				Next morning partly coagulated.		

The Action of Unheated and Heated Serum on Fluoride, Hirudin, and Peptone Plasma.

While tissue extracts are very active with hirudin plasma, blood serum is relatively little active.   With increasing quantities of serum the coagulating effect of serum becomes more marked. With fluoride plasma the curves of serum are often irregular. Small quantities are frequently as active as large ones; sometimes they are even more active, but in the majority of cases with increasing quantities of serum the effect becomes more marked. The same serum may show different curves with different kinds of fluoride plasma, and the optimal quantity producing the most rapid and complete coagulation may likewise differ.

Formerly we found in many cases that the homologous kind of serum acts relatively better than the heterologous serum with fluoride plasma, while with hirudin plasma usually the opposite relation prevails, the heterologous serum being relatively more potent than the homologous serum   We found again the same relation in these experiments, although it is not apparent in every case.   The result may vary with the amount of serum used. With a larger quantity a serum may be more effective with one kind of HPl, and with another quantity with another kind; and the same may hold good with DFlPl.   Using 1 cc. of serum DS may be better than GS, and with 0.5 cc. of serum GS may be better than DS.   With increasing quantities the inhibiting substance gains relatively more in GS.

Provisionally we could explain these relations if we assume[1] that in each serum there is a coagulating, as well as an inhibiting, substance.   Both are specifically adapted to its class homologous plasma.   With fluoride plasma the coagulating substance is of more importance, with the hirudin and peptone plasma, the inhibiting substance; but with increasing quantities the coagulating substance asserts itself ultimately in the hirudin and peptone plasma.   Different sera may differ as to the absolute quantities, as well as in relative proportions of coagulating and inhibiting substances.   In consequence of such variations a specific adaptation between serum and plasma may be observed.   Thus, if in a RS the coagulating substance happens to be absolutely greater and the inhibiting substance smaller than in DS and GS, then this particular RS might be more active with RHPl than DS and

GS, and a possible specificity of inhibiting substance might not be apparent. In one case GS did not show a relatively greater potency with the heterologous HPl; at the same time it was also quite inactive with FlPl. This might be interpreted as due to a relative lack of both coagulating and inhibiting substances in this particular serum. DS and GS may behave similarly with DHPl as with DFlPl, in case the inhibiting and accelerating substances balance each other.

The Effect of Heating on Serum.

In various experiments dog or goose serum was heated to 56 or 57° for a period of 15 or 30 minutes. Such sera, either uncombined or in combination with extracts, were compared with unheated sera in their action on fluoride and hirudin or peptone plasma. DS or GS which had been heated for 15 or 30 minutes had lost its power to cause coagulation of hirudin and peptone plasma. Heating the serum for 30 minutes destroyed the coagulating power towards fluoride plasma completely or almost completely. Some small remnants may occasionally be left. Especially GS in its effect on FlPl seems to be slightly less affected through heating than does DS. With serum heated for 15 minutes the results were more variable. There was always some loss, but sometimes it was almost complete, in other cases very marked, and in two cases only moderate. In the latter cases the result varied also with different kinds of fluoride plasma.

The acceleration in coagulation of DFlPl which occurs on mixing serum and extract and adding it at once to FlPl is usually still present after heating serum for 15 or 30 minutes, but it is diminished, and more so through heating for 30 minutes than for 15 minutes. We have, of course, to consider the possibility that calcium present in the heated, as well as in the unheated, serum may have a slight effect in accelerating the coagulation of the FlPl.

The presence of the inhibiting substance in serum becomes clear if we add the mixture of serum and extract at once to hirudin or peptone plasma, or if we allow the mixture to stand for variable periods before adding it to HPl, PPl, or FlPl. If we add the mixture of heated serum and extract to hirudin and peptone plasma, at once, we obtain the same results as in the case of

unheated serum. There is usually absent an activation; there may be an inhibition of the coagulating power of the extract after addition of heated serum. This inhibition may be even more marked in the case of the heated than of the unheated serum. In one experiment the coagulating power of DS as well as of GS in a mixture with TE was increased through previous heating for a period of 15 minutes.

In addition DS was improved through heating it for 30 minutes, but in the case of GS heating it for 30 minutes caused a deterioration and an increase in its inhibiting effect on TE. We can explain these results if we assume that both an inhibiting and coagulating substance are injured through heating and that according to the different degree in which each of these substances has been injured the effect of heating may vary. It is possible that the inhibiting action exerted by serum in the mixture, if this is added at once to the plasma, is of a complex nature; it may depend in part on a chemical combination between the inhibiting component of the extract and that of the serum, and in addition there may come into play an injurious physical effect of the serum colloids on the extract. Both effects may be influenced in a different way by the heating. The inhibiting effect of the heated serum may become distinct only if a larger quantity of serum (0.7 cc. of serum to 1 cc. of plasma) is added, while with 0.5 cc. of serum the inhibiting effect may not be apparent.

If we let the mixture of heated serum and extract stand for 6 minutes before adding it to the HPl or PPl, we find in general a great decrease in the inhibiting power of the serum. Again in using relatively small quantities of heated serum (0.4 cc.) it might appear as if the inhibiting substance had almost entirely disappeared, but if we use as much as 0.7 cc. it becomes apparent that the inhibiting substance has only been partially destroyed. Heating the serum during a period of 30 minutes destroys a greater part of the inhibiting substance than heating it for 15 minutes. Through the heating of the serum the time curve of the mixture of DS+DKE with DHPl or DPPl becomes somewhat similar to that observed in the case of FlPl, or to the time curve of a mixture of GE and TE in combination with HPl. The decrease in coagulating power on standing of the mixture of TE with the

heated serum (15 seconds) approaches more or less proportionately to the time during which the mixture had been standing. After heating the serum for 30 minutes the curve may be even flatter than would correspond to proportionality with the time of standing.

With DFlPl we find corresponding conditions. We have seen that if we add at once the mixture of the heated serum with the extract to the plasma, there is a decrease in activation. But at the same time the inhibiting substance is also partially destroyed through the heating; therefore, the loss after standing of the mixture for 6 minutes causes less deterioration than we find if unheated serum had been mixed with the TE. Yet there is still some loss after standing as compared to the coagulating effect of the mixture if added at once. Both the coagulating effect, seen if the mixture is added at once, and the inhibiting effect, seen if the mixture is added after standing, are diminished as the result of the heating; we find, therefore, that the coagulation times of the plasma, to which the mixture with the heated serum had been added at once, or after standing, may decome very similar to each other; they differ less than in the mixtures with unheated serum, and furthermore, the coagulation times of the FlPl to which the mixture has been added at once and after 6 minutes standing, are more alike after heating the serum for 30 seconds than for 15 seconds.

In one experiment a mixture with DS heated for 15 and 30 seconds caused even a slightly more rapid coagulation than when the mixture with heated serum had been added at once.

In this case we must assume that the inhibiting substance had been injured relatively more through heating than the activating substance. In the case of heated GS the destruction of inhibiting and activating substance just balanced each other in the same experiment.

The quantity curve of the serum shows a change parallel to the time curve if we substitute heated for fresh serum. There is with hirudin plasma still an increase in inhibition with an increase in quantity of serum, but the increase is more gradual and the curve less steep owing to the partial destruction of the inhibiting substance in the serum as the result of the heating.

The Action of Heated Serum on Plasma.

Mixture.	Coagulation time.
	hrs. min. sec
1 cc. DFPl + 1 cc. DKE	38 15
1 " " + 0.5 " "	47 Partly. Next morning partly coagulated.
1 " " + 1 " DS	9 5
1 " " + 0.7 " "	11 40
1 " " + 0.3 " "	19
1 " DHPl + 0.3 " DKE	4 25
1 " " + 1 " DS	Next morning not coagulated.
1 " " + 2 " "	6 28 Trace. Next morn ng almost coagulated.
1 " " + 1 " heated DS (56°)	Next morning not coagulated.
1 " " + 2 " " " (56°)	Next morning not coagulated.
1 " DFlPl + 1 " " " (56°)	Next morning trace.
1 " " + 0.7 " " " (56°)	" " "

Time of standing of S + E.	Mixture.	Coagulation time.	
min.		*min*	*sec.*
0	1 cc. DFPl + 0.7 cc. DS + 0.3 cc. DKE	2	55
½	1 " " + 0.7 " " + 0.3 " "	2	5
6	1 " " + 0.7 " " + 0.3 " "	5	
0	1 " DFPl + 0.7 cc. heated DS (56°) + 0.3 cc. DKE	5	45
e	1 cc. DFPl + 0.7 cc. heated DS (56°) + 0.3 cc. DKE	6	10
0	1 cc. DHPl + Pl + 0.7 cc. DS + 0.3 cc. DKE	8	
6	1 " " + " + 0.7 " " + 0.3 " "	Next morning trace.	
0	1 cc. DHPl + 0.7 cc. heated DS (56°) + 0.3 cc. DKE	10	50
6	1 cc. DHPl + 0.7 cc. heated DS (56°) + 0.3 cc. DKE	26	13 Partly. Next morning, almost coagulated.
0	1 cc. DHPl + 0.5 cc. DS + 0.5 cc. DKE	3	45
6	1 " " + 0.5 " " + 0.5 " "	Next morning, almost coagulated.	
0	1 cc. DHPl + 0.5 cc. heated DS + 0.5 cc. DKE	3	25
6	1 " " + 0.5 " " " + 0.5 " "	4	30
0	1 cc. DPPl + 0.5 cc. DS + 0.5 cc. DKE	2	10
6	1 " " + 0.5 " " + 0.5 " "	Next morning, almost coagulated.	
0	1 cc. DPPl + 0.5 cc. heated DS + 0.5 cc. DKE	2	15
6	1 " " + 0.5 " " " + 0.5 " "	2	40
0	1 cc. DFlPl + 0.7 cc. DS + 0.3 cc. DKE	3	20
6	1 " " + 0.7 " " + 0.3 " "	5	10
0	1 cc. DFlPl + 0.7 cc. heated DS + 0.3 cc. DKE	7	40
6	1 " " + 0.7 " " " + 0.3 " "	7	40

SUMMARY.

1. The effect of a mixture of serum and tissue extract on fluoride plasma, and the curve representing the coagulation time of the plasma as a function of the time during which the serum and extract were allowed to stand at room temperature before being added to the plasma, can be explained if we assume that two processes go hand in hand in the mixture; namely, the formation of a substance accelerating the coagulation of the plasma and of a substance inhibiting in some way the action of the coagulating substance.   The character of this curve varies with and depends upon the kind of extract and serum which are combined and on the relation of both serum and extract separately with the plasma.   We may, furthermore, assume that in the case of fluoride plasma, the serum component of the mixture is more important than the extract component.

2. With hirudin or peptone plasma the inhibition exerted by serum on the extract is very great.   Even if the mixture is added to the plasma at once, there is often some loss, although in other cases there may be a slight acceleration.   The loss increases rapidly with the time during which the mixture of DS+DKE is allowed to stand, until at last the coagulation is indefinitely prevented.   The optimum in coagulating power which is characteristic of the fluoride plasma curve is usually absent when the hirudin plasma is used.   Different combinations of extract and serum show different degrees of loss and different time curves. These curves seem to be characteristic for certain combinations and they correspond to those found in fluoride plasma; these variations depend on the character of the serum as well as of the extract.   With an increasing amount of serum in the mixture the inhibition increases markedly.   The curves representing the coagulation times as functions of the amount of serum in the mixture, correspond to the time curves with the same kind of serum.   If DS is used in the mixture both curves are very steep and there is a sharp turning point.   In a serum (RS) which, in the usual quantities added to the mixture, shows only slight inhibition with dog hirudin plasma, the inhibiting action may become very marked, when the amount of serum is much increased. The curves indicating the loss on standing of the various kinds of mixtures vary also with the kind of hirudin plasma used.

3. While with hirudin and peptone plasma the coagulating effect of serum increases with increasing quantities of serum, with fluoride plasma there may occur some deviations from this rule. While with fluoride plasma the homologous serum is often more active than the heterologous serum, with hirudin and peptone plasma the heterologous serum is more often more potent.   The potency of different sera may, however, change with the quantities used.

Heating the serum to 56° for 15 or 30 minutes destroys or weakens its coagulating action on hirudin and fluoride plasma. It weakens the acceleration of a mixture of serum with tissue extract, if this mixture is added at once to fluoride plasma but does not greatly alter the effect of such a mixture added at once to hirudin plasma.   It diminishes the loss which such a mixture experiences on standing before being added to the fluoride and peptone plasma.

We can interpret these facts if we assume that there are present in serum one or more components which tend to accelerate the coagulation of plasma, and another component which tends to inhibit it.   The accelerating component prevails with the fluoride plasma and with the hirudin and peptone plasma if large quantities of the latter are used.   An accelerating component likewise prevails in a mixture of serum and extract if this mixture is added at once to the fluoride plasma.   In all other cases, and especially after standing of the mixture for a short time, the inhibiting substance prevails.   Heating serum to 56° injures both the accelerating and inhibiting substances.   The effect of heating the serum varies, therefore, under different conditions in accordance with the relative importance of the accelerating and inhibiting functions of the serum under these conditions.

THE INTERACTION BETWEEN BLOOD SERUM AND TISSUE EXTRACT IN THE COAGULATION OF THE BLOOD.

II. A COMPARISON BETWEEN THE EFFECTS OF THE STROMA OF ERYTHROCYTES AND OF TISSUE EXTRACTS, UNHEATED AND HEATED, ON THE COAGULATION OF THE BLOOD, AND ON THE MECHANISM OF THE INTERACTION OF THESE SUBSTANCES WITH BLOOD SERUM.

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Curve of Tissue Extract with Hirudin Plasma and Fluoride Plasma.

If we add to tubes containing 1 cc. of HPl variable quantities of TE, we do not find the coagulation time to vary in an inverse ratio proportional to the quantity added, but instead irregular curves are found. In the majority of cases a medium quantity of TE represents the optimum; increasing the quantity above the optimum caused a decrease in activity or leaves the latter unchanged. Below the optimum the effect is weaker. In some cases, however, the largest quantities used (1 cc. of TE) were found optimal. The optimal quantity varies with the same extract, if different plasmas are used, and with the same plasma if different extracts are used. When after standing the extract loses in strength, and the optimal quantity loses, for instance, about 100 per cent, large quantities may be found inactive. In some cases we observed with extract, as with red blood corpuscles, that with a homologous plasma the optimum is lower than with a heterologous plasma. With FlPl we find a similar condition, but with FlPl 1 cc. is more often optimal or near the optimal point than with HPl; but even with FlPl smaller quantities may sometimes be as good or better than larger quantities.

Specificity of Tissue Extract.

In former experiments in which the tissue extracts were allowed to act directly on the unaltered blood or plasma, a general (class) specific adaptation between tissue coagulins and plasma was shown to exist.[1] Under certain conditions this specific adaptation can also be demonstrated if we use hirudin and fluoride plasma instead of fresh blood or plasma. In all cases it is necessary to make cross-tests in order to eliminate variations in the absolute strength of extracts; it is furthermore necessary to use extracts and plasma in various proportions. With HPl it is much more readily possible to demonstrate the specific adaptation than with FlPl, because with HPl the extracts alone usually cause coagulation of the plasma, while with FlPl, especially GFlPl, extracts alone are in some cases quite ineffective and thus it is impossible to prove the specific adaptation of extract and plasma. In such cases it can, however, sometimes be shown to exist by combining extract with serum. The homologous extract in combination with serum proves then in cross-tests relatively more active than heterologous combinations. In other cases the addition of serum to extract may, however, obscure the specific relationship between extract and plasma. This is due to the fact that in the mixture of serum and extract inhibiting substances are produced which counteract the tissue coagulins. ·

The Effect of Heating on Tissue Extract.　　.

Tissue extract was heated to 56–57° or to 60° generally for a period of 30 minutes, or in some cases for 15 minutes. The effect of DKE heated to 56° for 30 minutes on DHPl or DPPl depended in some cases on the quantity of DKE used. 0.3 cc. of DKE (added to 1 cc. of HPl) was usually rendered more active through heating, while 1 cc. of DKE remained either unchanged or was even weakened. The improvement in 0.3 cc. of DKE was in some cases slight, but in other cases considerable. Sometimes an improvement was produced through heating without a definite change in the character of the quantity curve. When-

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[1] Loeb, L., *Montreal Med. J.*, 1903, xxxii, 507; *Med. News*, 1903, lxxxiii, 212; *Virchows Arch. path. Anat.*, 1904, clxxvi, 10.

ever the smaller quantity was more improved ·through heating than the larger quantity, the quantity curve was altered through heating and sometimes the relative effects of large and small quantities were reversed through heating.

In an experiment with GME 1 cc. was improved through heating, but not 0.3 cc. if we tested the coagulating power of the extract with DHPl. With GHPl no improvement was produced through heating GME, but in this case also the larger quantity became, through heating, relatively better than the smaller quantity. With DLE (tested with DHPl) 0.3 cc. remained unchanged through heating, but 1 cc. was slightly weakened. While with unheated extracts DLE was more effective than DKE, after heating the relation was reversed, DKE being more effective than DLE.

We may explain these facts if we assume that in the extract at least two substances are present, one causing acceleration when added to plasma, the other inhibition. In different extracts these two substances are present in different proportions; in DKE there is a relatively larger quantity of inhibiting substance than in DLE. The relative preponderance of the two substances may also vary in different quantities. In the case of DKE the inhibiting substance happened to be relatively more potent in 0.3 than in 1 cc.; while in GME the reverse relation happened to hold good.

Heating the extract to 56° for 30 minutes injures the inhibiting substance to a higher degree than the coagulating substance and thus under certain conditions the coagulating power of the extract may be improved through heating. But heating injures not only the inhibiting substance but also the coagulating substance and thus in certain cases heating may fail to produce an improvement or may even cause a deterioration.

Heating DKE to 60° for 30 (or even 15) minutes weakens its coagulating power if tested with DHPl, quite definitely. Larger quantities were in our experiments more affected than smaller quantities. Heating DKE to 60° likewise weakens its coagulating effect on DPPl. We may assume that in heating to 60° the injury to the coagulating factor predominates over the weakening of the inhibiting substance.

We carried out also a few experiments in which we compared the action of heated and unheated extract on DFlPl.   Heating the DKE to 60° weakened it very much; heating it to 56° weakened it in two cases while in one case it produced a very slight improvement.   We may assume that with FlPl as with HPl heating injures both the inhibiting and coagulating substances, but with FlPl the inhibiting substance is of less importance than with HPl and PPl, and that, therefore, the weakening of the coagulating substance caused through heating counts in this case more than with HPl and PPl, or at least both the inhibiting and coagulating substance balance each other more nearly in the case of FlPl.

In combination with serum the effect of heating on extract corresponds to the effect of heating on the uncombined extract if we use DHPl or DPPl to test the coagulating power.   As we stated the coagulating effect of extract on DHPl or DPPl is usually increased through heating.   Correspondingly we find that the inhibiting effect of S on DKE is usually diminished or entirely removed through heating the extract to 56° for 30 minutes; or instead of an inhibition we may even find a slight acceleration of the mixture as compared with the extract alone provided the mixture of extract and serum is added to the plasma at once.   In the majority of these experiments 0.3 cc. of extract was mixed with 0.7 cc, of serum and the mixture added at once to 1 cc. of HPl or PPl.   In case the heating does not improve the coagulating effect of the uncombined TE, the inhibiting effect of serum in combination with such heated extract, if added at once to the plasma, is not diminished.   This occurred, for instance, in our experiment with DLE.

If the mixture of heated extract (56°) and serum is allowed to stand 6 minutes before being added to the plasma, an inhibiting effect of the serum is still noticeable, but the loss in the mixture of DKE + S on standing is much reduced through the heating of the extract.   While with the unheated DKE the mixture usually loses many times in efficiency, the loss of the mixture of heated extract and serum was much smaller and varied in our experiments between 150 and 300 per cent.   The time curve in the mixture acting on DHPl or DPPl has, therefore, become not unlike that of the mixture of unheated E + S when acting on

DFlPl. A great part of the inhibiting effect on standing has been eliminated through heating. Quantitative variations in the diminution of the inhibiting effect through heating do, however, occur and in one case the diminution in the loss of the mixture after standing was less than usual. In this case DPPl had been used. In cases in which the inhibiting substance in the extract plays a smaller part as in a mixture of DLE + DS, the time curve is not very much changed through the heating; the effect of the heating on the time curve is likewise diminished, if we decrease the amount of serum added to the mixture, because a constituent of the serum is necessary for the production of the inhibiting effect. The more the serum is diminished in experiments with HPl or PPl, the less becomes the inhibiting effect, and the less significant becomes the effect of heating on the extract.

If we test with DHPl or DPPl an extract-serum mixture in which the extract has been heated to 60° for half an hour, the mixture resembles in its action much more a mixture of unheated extract and serum. This holds good if the mixture is added at once to the plasma as well as when it is added after a previous standing for a period of 6 minutes. In other cases the effect of the mixture of DKE + DS, in which the DKE had previously been heated to 60°, is intermediate between that of a mixture of unheated DKE+DS and of DKE+DS in which the DKE had been heated to 56°, but usually it is nearer the former.

We may interpret these facts by assuming that through heating to 60° the coagulating component of the TE is injured to approximately the same extent as the inhibiting substance or only slightly less; and that thus through heating to 60° the balance between coagulating and inhibiting substance is always less favorably affected than through heating to 56°. The gain shown through the heating to 56° is, therefore, again lost or at least diminished through heating the extract to 60°.

If we test the effect of the mixture of 56° heated DKE+DS with DFlPl instead of with DHPl and DPPl, and if we add the mixture to the plasma at once, we usually find some improvement as compared to the effect of the mixture of unheated DKE+DS on DFlPl; in some cases no change in the efficiency of the mixture takes place through the heating.

The time 'curve of the 56° heated DKE+DS with DFlPl is usually similar to that of the unheated DKE+DS; there is, therefore, usually some loss after 6 minutes standing which is, however, considerably smaller than in the case of DHPl or DPPl. But inasmuch as there is usually some gain, if the mixture of 56° heated DKE+DS is added at once to the DFlPl, there is also, after 6 minutes standing, usually some absolute gain in the coagulating effect of the 56° heated DKE+DS mixture as compared to the mixture of unheated DKE+DS. Even 56° heated DLE may be better in combination with DS as compared to unheated DLE in combination with DS, although with DHPl, and if used uncombined with DFlPl, heating did not improve DLE in the experiment in which we used it.

A mixture of 60° DKE+DS added to DFlPl had less coagulating power than a mixture of unheated DKE+DS. The injury to the coagulating substance caused through heating DKE to 60° is more evident in the case of DFlPl than in the case of DHPl and DPPl. In the case of the latter the fate of the inhibiting substance is of relatively greater importance.

The Effect of Heated Extract on Hirudin and Fluoride Plasma.

Mixture.	Coagulation time.		
	hrs.	min.	sec.
1 cc. DHPl + 1 cc. DKE		5	25
1 " " + 0.3 " "		14	30
1 " " + 1 " heated (56°) DKE		5	40
1 " " + 0.3 " " (56°) "		5	30
1 " " + 1 " GME	5	13	
1 " " + 0.3 " "	1	57	
1 " " + 1 " heated (56°) GME	2	13	
1 " " + 0.3 " " (56°) "	5	9	

Time of standing of E + S.	Mixture.	Coagulation time.
min.		min. sec.
0	1 cc. DHPl + 0.7 cc. DS + 0.3 cc. DKE	Coagulated next morning.
0	1 " " + 0.7 " " + 0.3 " heated (56°) DKE	6 40
6	1 cc. DHPl + 0.7 cc. DS + 0 3 cc. DKE	Partly coagulated next morning.
6	1 " " + 0.7 " " + 0.3 " heated (56°) DKE	21 10
0	1 cc. DHPl + 0.7 cc. GS + 0.3 cc. GME	Trace coagulated next morning.
0	1 " " + 0.7 " " + 0.3 " heated (56°) GME	Not coagulated next morning.
6	1 cc. DHPl + 0.7 cc. GS + 0.3 cc. GME	" " "
6	1 " " + 0.7 " " + 0 3 " heated (56°) GME	" " "
6	1 cc. DFlPl + 0.7 cc. DS + 0.3 cc. DKE	Next morning coagulated.
6	1 " " + 0 7 " " + 0 3 " heated (56°) DKE	31 15

Mixture	Coagulation time.		
	hrs.	min.	sec.
1 cc. DHPl + 1 cc. DKE		1	25
1 " " + 0 3 " "		5	15
1 " " + 1 " heated (56°) DKE		1	35
1 " " + 0.3 " " (56°) "		2	
1 " " + 1 " " (60°) "		12	
1 " " + 0.3 " " (60°) "		8	15
1 " DFlPl + 1 " DKE		12	55
1 " " + 0.3 " "	1	12	Partly coagulated; next morning partly coagulated.
1 " " + 1 " heated (56°) DKE		14	55
1 " " + 0.3 " " (56°) "		29	45 Almost coagulated.
1 " " + 1 " " (60°) "		30	45 Mostly coagulated.
1 " " + 0.3 " " (60°) "	Trace coagulated next morning.		

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Time of stand-ing of D + S.	Mixture.	Coagulation time		
min.		hrs.	min	sec
0	1 cc. DHPl + 0 7 cc. DS + 0.3 cc. DKE		30	30
		Almost coagulated.		
6	1 " " + 0.7 " " + 0.3 " DKE	Next morning. Trace coagulated.		
0	1 " " + 0.7 " " + 0.3 " heated (56°) DKE		2	35
6	1 cc. DHPl + 0.7 cc. DS + 0.3 cc. heated (56°) DKE		6	15
0	1 cc. DHPl + 0.7 cc. DS + 0.3 cc. heated (60°) DKE		16	40
6	1 cc. DHPl + 0.7 cc. DS + 0.3 cc. heated (60°) DKE	2	9	Trace.
		Next morning partly coagulated.		
0	1 cc. DFlPl + 0.7 " " + 0.3 cc. DKE		2	45
6	1 " " + 0 7 " " + 0.3 " "		6	
0	1 " " + 0.7 " " + 0.3 cc. heated (56°) DKE		2	5
6	1 cc. DFlPl + 0.7 cc. DS + 0.3 cc. heated (56°) DKE		4	5
0	1 cc. DFlPl + 0.7 cc. DS + 0.3 cc. heated (60°) DKE		5	40
6	1 cc. DFlPl + 0.7 cc. DS + 0.3 cc. heated (60°) DKE		11	50

The Effect of Unheated and Heated Stroma of Erythrocytes with or Without the Addition of Serum.

A suspension of the stroma of red corpuscles was obtained in the following manner: The corpuscles of defibrinated blood were separated through centrifugation from the serum and washed four times with 0.85 per cent NaCl solution. The corpuscles of 100 cc. of blood were then suspended in 76 cc. of salt solution and an equal amount of distilled water was added. After standing the stroma was separated from the hemoglobin through centrifugation. To the suspension of stroma so much NaCl was added that a 0.85 per cent solution resulted.

We found the curves of TE with hirudin, peptone, and fluoride plasma representing the coagulation time as a function of the amount of TE to be irregular. The same holds good in the case of stroma of erythrocytes (Cp). The character of the curve depends to some extent upon the species whose corpuscles were used; and even the corpuscles obtained from different individuals may differ in some respects from each other. It also depends upon species and individual variations in the plasma. There is an optimum for each Cp preparation below and above which it is less active. With 1 cc. of DHPl the optimum is in many cases between 0.4 and 0.7 cc. of RCp, but it may be as high as 1 cc. With DCp and CCp it is often between 0.2 and 0.4 cc. The curve may be flat or show a sharp turning point. With RCp it is on the whole flatter than with DCp or CCp. The latter two may, for instance, show a sharp turning point between 0.7 and 1 cc. With the little active turtle corpuscles larger quantities are best. Also GCp are absolutely less active than mammalian Cp. Cp may cause coagulation not only of the HPl and PPl but also of FlPl, but less actively than serum; in this respect they again resemble TE. The effect depends in this case also on the kind of plasma used. With DFlPl DCp may be better in smaller quantities; with RFlPl both large and small quantities may be equally active; and with GFlPl larger quantities may be better. Also with different samples of DHPl the optimum of DCp and RCp may vary and both may vary in the opposite direction. In several cases the optimum varied with different plasmas in such a way that with the same kind of Cp the optimum was lower with the homologous than with the heterologous plasma.

Through cross-tests between Cp and plasma it is not only possible to establish a relative class specificity, as in the case of TE, but also a specific species adaptation between plasma and corpuscles.[2] Dog, cat, rabbit, and goose corpuscle act relatively best each with its own plasma. While, therefore, GCp are absolutely little active, relatively they show the greatest coagulating power in combination with GPl.

We compared the action of Cp on DHPl and DPPl.[3] Cp

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[2] Loeb, L., and Fleisher, M. S., *Biochem. Z.*, 1910, xxviii, 169.

[3] While the tests made with DPPl were numerous and concordant with each other, the specimens of PPl used in our experiments had all been obtained from one individual dog. It would be desirable to make additional tests with the PPl from different individuals.

was found to be much less effective towards DPPl than towards DHPl.   DTE, on the other hand, was about as active with DPPl as with DHPl; often it was more active with DHPl and only once was it found to be slightly less active.   Also in combination with serum Cp was relatively inactive towards DPPl as compared with DHPl.   TE + S, on the other hand, is at least as active towards DPPl as against DHPl.   DS and RS, unheated and heated, behaved alike towards both kinds of plasma. A combination of S with Cp, if added at once to DPPl, may cause as much acceleration of the coagulation as corresponds to a summation of the individual effects of Cp and S on DPPl With S alone DHPl coagulated somewhat better than DPPl.

This greater responsiveness to Cp of DHPl as compared with DPPl may also influence the optimum of Cp.   In one experiment, for instance, with DPPl 1 cc. of Cp was better than 0.3 cc.; while with DHPl, on the contrary, 0.3 cc. was better than 1 cc.   It often holds good that the more inactive the Cp is, the higher relatively is their optimum.

As we have seen, a mixture of S and TE, if added at once to HPl or PPl, is usually less active than TE alone; the mixture added at once to FlPl usually shows activation.   After standing of the mixture, there is a loss in efficiency with all three kinds of plasmas, but it is much more marked with HPl and PPl than with FlPl.'   While Cp behaves essentially like TE, there is a marked difference between the behavior of Cp + S on the one hand of TE + S on the other hand towards the three kinds of plasma.

A mixture of Cp + S, added at once to HPl, is usually as active as Cp alone; or sometimes it is slightly more active. In a few cases it was even markedly more active, and in a few other cases there was just an indication of a loss as compared with the Cp alone.   After 6 to 10 minutes standing, the mixture has in most cases gained in coagulative power, even in cases in which if added at once it had not been more active than the Cp alone.   This improvement may be slight or marked.   There are cases in which there is no improvement, or even a slight loss in the coagulative power of the mixture on standing.   The time curves of the mixture vary in different cases and the effect de-

pends on the time when the mixture has been added to the HPl. The gain in power through standing may extend over a period of several hours, though the optimum is usually reached at an earlier period; this optimum is followed by a loss which is, however, very gradual.   The shortening of the coagulation time with the standing of the mixture may be progressive up to a period of 30 minutes.   In other cases the optimum is reached after 5 to 10 minutes standing, and after 30 minutes standing there is again some decrease which has become greater after 3 hours standing. In one case there was no improvement in the mixture, when added at once; after 6 minutes standing there was a slight improvement and after 10 minutes there was again a slight loss.   The improvement in the mixture may already be very marked if it is added at once, while after 10 minutes standing there may still be some improvement, although less than when the mixture has been added at once.   In some cases there was no improvement in the mixture, either when added at once, or after 6 minutes standing. With DFlPl the improvement caused through mixing Cp and S as compared with Cp or S alone, is usually greater than with DHPl if the mixture is added at once to the plasma.   The same effect was found in the mixture of S + TE.   In some cases, however, there was no activation through the mixing of S and DKE, when adding the mixture at once to DFlPl.   After 6 to10 minutes standing of Cp + S, there may be great improvement over the mixture added at once; this applies to cases in which the mixture, if added at once, did produce an acceleration as well as to other cases in which it did not produce an acceleration.   In other cases, however, there was not found any improvement or even a slight deterioration after 6 minutes standing.

If we compare the mixture of the same kind of Cp and S with HPl and FlPl in a particular case, we find corresponding time curves with both kinds of plasma and the optimum is situated at similar points of the curve, although the absolute quantities of acceleration differ, the improvement being usually greater with DFlPl.

If, on the other hand, we compare the mixtures of Cp and S in their action on HPl and FlPl of different species, we may find differences in the curves.   With DHPl and RHPl the curves may differ; with DS there may be after 6 minutes standing more loss with DCp than with RCp.   Those kinds of Cp which alone

are most active with HPl usually accelerate also most effectively in combination with serum.

Effect of Heated Cp.—While tissue extract heated to 56° is often more effective in causing coagulation than unheated extract, such an improvement is not found after heating Cp to 56° for 30 seconds.

Effect of Heated Cp on DHPl.—There is uniformly a loss in the coagulating power of the Cp when uncombined with serum. This loss usually varies between 50 and 300 per cent, but it may be much greater (1,200 to 2,000 per cent). In cases in which the unheated Cp is more active in the smaller quantity (0.3 cc.), heating may in some cases cause a reversal and make the larger quantity (1 cc.) more active than the smaller one without, however, producing an absolute improvement. The same reversal we observed in the case of heated TE.

Inasmuch as even unheated Cp is relatively inactive with DFlPl, heating renders the uncombined Cp quite inactive with FlPl. A mixture of S + heated DCp acts on HPl as we might expect from a knowledge of the effect of a mixture of S + unheated Cp on HPl and of the effect of heating on the uncombined Cp. The combination of S + heated Cp, if added to HPl at once, is to the same extent less active than the mixture of S + unheated Cp as heated Cp alone is less active than unheated Cp. Or the loss caused by heating may appear even somewhat greater in combination than uncombined. Thus if the combination of unheated corpuscles with serum, added at once to the HPl, neither accelerates nor inhibits as compared with the action of unheated Cp alone, then the use of heated Cp may show a slight loss in combination as compared to the action of heated Cp alone. After 6 minutes standing S + heated Cp may be more effective than the same mixture added at once, as we found to be the case with the mixture of serum and unheated Cp; but the mixture with the heated Cp is distinctly less effective than S + unheated Cp after 6 minutes standing. Relatively it may gain as much as S + unheated Cp after 6 minutes standing or the gain may be somewhat less. On the whole the more the mixture of S + unheated Cp surpasses unheated Cp alone in effectiveness, if added to the HPl at once or after 6 minutes standing the more the mixture of S + heated Cp surpasses the heated Cp alone. There is, therefore, a parallelism between the behavior of the

heated and unheated Cp with HPl, but the heated Cp is always distinctly less effective.

With DPPl heated Cp alone as well as in combination with S may be quite inactive. This corresponds to the low degree of efficiency of Cp towards DPPl.

While with DFlPl heated Cp alone may be inactive, in combination with serum it may be active, but again less so than unheated Cp in˙combination with serum. Both unheated and heated Cp may gain approximately in a similar proportion after standing with serum for 6 minutes; though the gain of the heated Cp may be relatively slightly less than that of the unheated Cp. Absolutely the mixture of heated Cp + S is less active than the mixture with unheated Cp.

If we apply in the case of the Cp the same reasoning which we used in the case of TE, we may conclude from the fact that heating the corpuscles causes only loss and never a gain in coagulative power, that in the erythrocytes the inhibiting substance which we found in TE is absent or at least present in a much smaller quantity than in TE. This conclusion is corroborated by the fact that on standing with serum the Cp gains in coagulative power under conditions in which a combination of TE + S loses, and that the loss of a combination of S + Cp is much more gradual and much slower than the loss in a combination of S + TE. The gain usually is noticeable for a relatively long period of time in the combination of S + Cp. We may therefore conclude that the accelerating substance forms mainly in combination of Cp with S and that the inhibiting substance either does not form at all or forms in a much smaller quantity. On the other hand, we again find corroborative evidence for our conclusion that heating tissue coagulin to 56° injures it to some extent; this comes out much more clearly with Cp because here the destruction of an inhibiting effect does not obscure the injurious effect of moderate heat on the coagulating substance.

We may furthermore conclude that inasmuch as the irregular curves are found in the case of uncombined Cp, as well as of uncombined TE in their action on HPl, although in the former the inhibiting substance is present to a much smaller extent than in the TE, these irregular curves cannot be due, or at least cannot be entirely due, to the same inhibiting substance which combines with the serum.

Mixture.	Coagulation time.	
	min.	sec.
1 cc. DHPl + 0.3 cc. DCp	5	50
1 " " + 0.3 " CCp	4	
1 " " + 0.7 " DS	Next morning, no coagulation.	
1 " DFlPl + 0.3 " DCp	54	
1 " " + 0.3 " CCp	5	30
1 " " + 0.7 " DS	20	5

Time of standing of S + Cp	Mixture.	Coagulation time.	
min.		min.	sec.
0	1 cc. DHPl + 0.7 cc. DS + 0 3 cc. DCp	3	
5	1 " " + 0.7 " " + 0.3 " "	1	35
10	1 " " + 0.7 " " + 0.3 " "	1	50
15	1 " " + 0.7 " " + 0.3 " "	2	50
30	1 " " + 0.7 " " + 0.3 " "	5	30
hrs.			
3	1 " " + 0.7 " " + 0.3 " "	10	
min.			
0	1 " " + 0.7 " " + 0.3 " CCp	2	35
10	1 " " + 0.7 " " + 0.3 " "	2	5
30	1 " " + 0.7 " " + 0.3 " "	3	
0	1 " DFlPl + 0.7 " " + 0.3 " DCp	1	45
5	1 " " + 0.7 " " + 0.3 " "		35
10	1 " " + 0.7 " " + 0.3 " "		25
15	1 " " + 0.7 " " + 0.3 " "		28
30	1 " " + 0.7 " " + 0.3 " "	1	20
hrs.			
3	1 " " + 0.7 " " + 0.3 " "	4	50
min.			
0	1 " " + 0.7 " " + 0.3 " CCp		45
10	1 " " + 0.7 " " + 0.3 " "		35
30	1 " " + 0.7 " " + 0.3 " "		55
0	1 cc. DFlPl + 0.3 cc. DCp + 0.7 cc. DS	3	10
6	1 " " + 0.3 " " + 0.7 " "	2	20
0	1 " " + 0.3 " heated (56°) DCp + 0.7 cc. DS	8	15
6	1 " " + 0.3 " (56°) " + 0.7 " "	6	35
	1 " " + 0.7 " DS	4	10
	1 " " + 0.3 " DCp	13	20
0	1 " " + 0.7 " DS + 0.3 cc. DCp	3	20
2	1 " " + 0.7 " " + 0.3 " "	2	
10	1 " " + 0.7 " " + 0.3 " "	1	40
	1 " " + 0.3 " CCp	6	15
0	1 " " + 0.7 " DS + 0.3 cc. CCp	2	30
2	1 " " + 0.7 " " + 0.3 " "	1	25
10	1 " " + 0.7 " " + 0.3 " "		50
30	1 " " + 0.7 " " + 0.3 " "		45

Mixture.	Coagulation time.		
	hrs.	min.	sec.
1 cc. DHPl + 0.4 cc. DCp		32	10
1 " " + 0.3 " "		23	30
1 " " + 0.2 " "		20	40
1 " " + 0.4 " heated (56°) DCp	1	23	15
1 " " + 0.3 " " (56°) "		42	Beginning coagulation.
	5	16	Coagulated.
1 " " + 0.2 " " (56°) "		31	Beginning coagulation.
	5	16	Coagulated.

Time of standing of S + Cp.	Mixture	Coagulation time.	
min.		min.	sec.
0	1 cc. DHPl + 0.3 cc. DCp + 0.7 cc. DS	9	45
6	1 " " + 0.3 " " + 0.7 " "	9	55
0	1 " " + 0.3 " heated (56°) DCp + 0.7 cc. DS	38	
2	1 " " + 0.3 " " (56°) " + 0.7 " "	40	
6	1 " " + 0.3 " " (56°) " + 0 7 " "	40	

On the Mechanism of Inhibition.

We carried out a few additional experiments in order to determine more definitely the mechanism underlying the inhibiting effect or the loss in coagulating power which occurs when the mixture of serum and extract is allowed to stand for a short period of time. If instead of letting a mixture of 0.7 cc. of DS + 0.5 cc. of DKE stand at room temperature, we keep it on ice for 10 minutes and then add the mixture to 1 cc. of RHPl, the inhibition is very much diminished.· The mixture added to the HPl at once caused coagulation in $6\frac{1}{2}$ minutes; after 10 minutes standing at room temperature, the coagulation was prevented indefinitely. But after 10 minutes standing on ice, the coagulation time was 23 minutes. Some inhibiting substance had been formed, but it was much less than at room temperature. The

prolongation of the coagulation time, after the mixture had been standing on ice, was not entirely due to the formation or action of an inhibiting substance, but partly at least to the lowering of the temperature in the mixture which persisted for some time after the plasma had been added.   From this experiment we may conclude that very probably a chemical reaction takes place between the inhibiting constituents of the serum and of the tissue extract and that this reaction is retarded by the lowering of the temperature.

If a combination of DKE + DS after standing for a short time no longer causes coagulation of RHPl, the addition of 0.5 cc. of new DKE to the mixture of DHPl, DKE, and S causes coagulation, which occurs either promptly or with a slight delay. We may, therefore, conclude that in the DKE + DS on standing no marked excess of inhibiting substance had been produced such as would have been able to neutralize an additional amount of TE.

If instead of combining DKE with DS we use with the. DKE an amount of DFlPl equal to the DS in the usual mixture, and allow the mixture to stand for 7 minutes before adding it to the HPl, a retardation in the coagulation of the DHPl occurs which, in our experiment, amounted to 100 per cent and which was probably due to the inhibiting effect of the fluoride as such, but in contradistinction to the mixture of DKE + S, this inhibition does not increase through standing for 7 minutes of the mixture of DFlPl + DKE.   In this case the interaction between TE and the DFlPl led to the production of coagulating substance which counteracted the inhibiting substance which may perhaps have formed in a mixture of DKE + DFlPl on standing.

In another experiment we determined whether it is possible to withdraw from the serum the inhibiting substance by shaking it with the residue of finely divided kidney tissue which had previously been used for the extraction of tissue coagulins.   20 cc. of DS were shaken with this residue of kidney tissue for 1 hour.   Such serum when separated from the kidney particles and allowed to stand with fresh DKE during the usual time proved still very inhibiting.   The inhibiting substance had, therefore, not been removed from the serum through this procedure.

1. With hirudin as well as with fluoride plasma the curves representing the coagulation time as a function of the quantity of tissue extract added are irregular, a medium quantity usually being optimal. The specific adaptation of the tissue coagulins to the plasma can be demonstrated not only with unaltered blood or plasma, but also with hirudin plasma and in certain cases with fluoride plasma. The inhibiting effect of serum may in some cases obscure the specific adaptation of the tissue coagulins in a mixture of extract and serum.

2. Heating extract to 56° very often produces an increase in its coagulating power, if the extract is added to the plasma in certain proportions. This improvement in all probability is due to a decrease in the inhibiting power caused by a moderate degree of heat which is greater than the injury to the coagulating substance caused by heating. Those extracts are especially benefited through heating in which the inhibiting substance prevails in the unheated extract. Heating the extract to 60° injures its coagulating power more than its inhibiting power if it is used uncombined with plasma. In combination with serum an extract heated to 56°, if tested with hirudin or peptone plasma, also shows a great diminution in inhibiting effect which becomes especially distinct if the mixture of serum and heated extract are allowed to stand for a few minutes. The use of sera and extracts under conditions which relatively increase the importance of the inhibiting effect of the tissue extract and serum increase *pari passu* the beneficial effect of the heating of the extract. A mixture of serum and of extract heated to 60° tends to become similar to a mixture of serum and unheated extract. With fluoride plasma a mixture of serum and extract likewise becomes more efficient through heating the extract to 56°. Heating the extract to 60° diminishes the coagulating power of the mixture as compared with the mixture of serum and unheated extract. The differences between the effects of the mixtures on hirudin and fluoride plasma can be explained if we assume that the inhibiting substance formed is of relatively more importance with the hirudin and peptone plasma and the coagulating substance with the fluoride plasma.

3. The curves representing the coagulating power of the stroma of erythrocytes as a function of quantity are similar to those of

tissue extract; the curves of different species differ.   It is possible
not only to demonstrate a relative class specific adaptation, but
even a species specific adaptation between corpuscles and plasma.
While organ tissue extract and serum are about equally active
towards DHPl and DPPl, corpuscles were found much less active
with DPPl than with DHPl.

In contradistinction to what we found in the case of mixtures
of tissue extract and serum, mixtures of Cp and S usually show
an acceleration, if added at once to hirudin or fluoride plasma;
on standing there is in most cases a gain, and only very gradually
a decrease occurs in the coagulating power of the mixture.   There
is, therefore, a marked difference between the behavior of such
mixtures and mixtures of TE and S.   These differences can be
explained if we assume that the inhibiting substance which com-
bines with a component of the serum is lacking in erythrocytes,
or at least much weaker than in the tissue extract.   Thus the
opposite reaction which leads to an acceleration preponderates
for some time.   Heating corpuscle stroma merely injures the
coagulating substance, the tissue coagulin.   It does not lead to
an improvement as heating of the tissue extract does, because in
Cp the inhibiting component is absent or at best present only in
small amounts.   Our conclusion is confirmed that even a moder-
ate heating injures the tissue coagulin and that the apparent
lack of effect of heating is due to the simultaneous injury to an
inhibiting substance.   Through combination with serum, heated
stroma of corpuscles improves relatively to the same extent or
somewhat less than unheated corpuscles.   As in the case of
the unheated corpuscles, this improvement is still more marked
after the heated corpuscles have been standing for some time.
The coagulating power of the heated mixture is smaller than that
of the mixture with the unheated corpuscles.

Inasmuch as in the corpuscles the inhibiting substance is much
less prominent than in tissue extract, and yet the irregular curves
of the uncombined corpuscles in their action on plasma resemble
those of the uncombined tissue extract, these irregular curves
cannot be altogether due to the same inhibiting mechanism which
acts in combination with serum.

4. The evidence points to the conclusion that a chemical com-
bination forms between a constituent of the tissue extract and the

serum which leads to the inhibition in the coagulation of the plasma, and that a lowering of the temperature increases the reaction time.   Two processes are taking place simultaneously, if blood serum and extract are mixed.   (*a*) Interactions take place which lead to the formation of a substance accelerating the coagulation of the blood.   This is shown by the optimum in the time curve of hirudin and fluoride plasma, if stroma of erythrocytes is combined with serum, and in the optimum of the time curve of fluoride plasma if tissue extract is used in combination with serum. (*b*) An inhibiting substance is formed through the combination of a constituent of the serum and of the tissue extract.   Both these constituents are injured by a moderate degree of heat.   In different blood sera the quantity and character of this inhibiting substance vary.   It is usually present in large quantities in blood serum of the dog.   The quantity and presumably also the character vary apparently in the tissue extracts in different species, and there are also variations in different tissues of the same species. In the mammalian red blood corpuscles, this inhibiting substance is found, if at all, only in very small quantities, while in the kidney and probably also in the spleen of dog it usually is found in large quantities.   Preliminary experiments which ought to be extended seem to indicate that there are also variations in the strength of the inhibiting substance in other organs of the same species.   We find accordingly, if one kind of blood serum is combined and incubated with various kinds of extracts that the results vary and that certain combinations lead to the production of an inhibiting substance to a much higher degree than others.   Accordingly we find that those sera and extracts which are most inhibiting lose absolutely more of their inhibiting power through a moderate heating than sera or extracts which have less of the inhibiting substance.

We find, furthermore, that through a graded moderate heating of the tissue extract the coagulating power of the tissue extract alone, as well as of the combination of serum and tissue extract, can be increased.   This is due to the fact that the inhibiting substance is slightly more sensitive to moderate heat than the coagulating substance; the latter is, however, also distinctly heat labile.

Heating the serum diminishes both the quantity of the inhibiting and coagulating substances and usually an increase in the

coagulating power of the serum cannot be produced through graded moderate heating of the serum, although in a few experiments we have seen indications that possibly a slight improvement may be procurable under certain conditions.   Through graded heating it is possible to separate the tissue coagulin from the inhibiting substance.   We may interpret these facts by assuming that both in the serum and the tissue extract there are substances present which unite to form the inhibiting substance.   The substances in the uncombined serum and extract are apparently only the precursors of the active inhibiting substance.[4]

As to the mode of action of this inhibiting substance, whether in some way it combines with the newly formed thrombin and thus inactivates the latter, or whether it acts in other ways, must be left undetermined at present.

5. In our earlier experiments we concluded that the tissue coagulins are specifically adapted to a substance in the blood plasma, while in the serum no evidence of a specific adaptation of the coagulating substance can be found.[1]   Subsequently when we observed the formation of an inhibiting substance in the mixture of serum and extract, we pointed out that the presence of the inhibiting substance might possibly obscure the specific adaptation of both the coagulating and inhibiting substances in the serum and that there are indications which indeed suggest such a specific adaptation of both the accelerating and inhibiting substance.[5]   Our present results are quite compatible with such an interpretation, although further experiments are necessary in this direction.

6. Our experiments show that while the character of the substances admixed to the tissue coagulins varies greatly in different extracts and in the erythrocytes, the specific adaptation of the tissue coagulins is not altered thereby.   This fact makes extremely improbable the assumption that the specific adaptation of the tissue coagulins is due to the admixture of other substances.

[4] It would be very desirable to extend the study of the effect of graded heat to other extracts; we intend to carry out these and other experiments as soon as possible.

[5] Loeb, L., *Beitr. chem. Physiol. u. Path.*, 1904, v, 534; 1907, ix, 185.

We may draw the further conclusion that such a specific adaptation would not be compatible with the opinion of several investigators according to which the tissue coagulins are of a lipoid character.   From all we know at the present time such a definite specificity is only found in substances of a protein nature, and accordingly we have assumed that there must be present in the tissue coagulins a protein component; but inasmuch as the studies of Bordet and Delange,[6] Howell,[7] and Zak[8] undoubtedly proved a coagulating effect of lipoid substances, we formed the opinion that possibly the combination of a protein and lipoid substance might constitute the tissue coagulins.   The recent studies of Mills[9] likewise point to this conclusion.

7. While our experiments prove that an interaction between a constituent of tissue and blood serum leads to the formation of a substance accelerating the coagulation of the blood, and in so far confirms the conceptions of Morawitz[10] and others, the possibility is not thereby excluded that in addition there may be a direct interaction between the tissue coagulins and the coagulable substance of the plasma.   In invertebrate blood our studies have made such an interaction very probable and there undoubtedly exist very marked analogies between the coagulation of the invertebrate and vertebrate blood.[11] Furthermore, certain facts in the coagulation of vertebrate blood likewise suggest such an interpretation.   It is therefore possible that we have not only to deal with different kinds of active components in cells, such as the protein and lipoid constituents, but even with different processes whereby the tissues accelerate the coagulation of the blood.

8. It is well known that coagulating substances are not only found in the blood serum and tissue extracts, but also in other material.   Thus we found previously a very strong coagulating substance in cultures of the staphylococcus which is destroyed

[6] Bordet, J., and Delange, L., *Arch. exp. Path. u. Pharmakol.*, 1913, lxxi, 293.

[7] Howell, W. H., *Am. J. Physiol.*, 1912–13, xxxi, 1.

[8] Zak, E., *Arch. exp. Path. u. Pharmakol.*, 1912, lxx, 27.

[9] Mills, C. A., *J. Biol. Chem.*, 1921, xlvi, 135.

[10] Morawitz, P., *Deutsch. Arch. klin. Med.*, 1903–04, lxxix, 1; *Beitr. chem. Physiol. u. Path.*, 1904, v, 133.

[11] Loeb, L., *Biochem. Centr.*, 1907, vi, 829.

by heat.[12]   It would be of interest to determine whether inhibiting substances in the blood serum and tissue extract are able to interact also with these substances.

In regard to the inhibiting component of the serum, we have made it very apparent that it disappears from the blood as a result of phosphorous poisoning.[5]   In the tissues we found previously fibrinolytic substances; whether or not they have some relation to the inhibiting component of the tissue extract is uncertain.[13]

<div align="center">CONCLUSIONS.</div>

The tissues contain constituents which can combine with a substance in the blood serum and thus lead to the production of a substance inhibiting the coagulation of the blood.   This substance differs in its action on fluoride plasma on the one hand, and on hirudin and peptone plasma on the other.

The quantity of these substances present in various kinds of cells varies greatly and they seem to be either absent or present only in small quantities in the erythrocytes.   These substances on the presence of which depends the inhibiting effect of the blood serum can be separated from the tissue coagulins through graded heating; the tissue coagulins, as well as the inhibiting component of the tissues, are injured through moderate heat.   Both the inhibiting component in the blood serum and that in the tissues seem to be similarly affected by heat.

The specific adaptation of the tissue coagulins remains constant, however much the inhibiting substances in the cells may vary in quantity.   This characteristic resides, therefore, in all probability in the tissue coagulins proper and is not dependent on admixtures.

The specific adaptation of the tissue coagulins makes it very probable that a protein component is concerned in the coagulating effect of the tissue extracts.

[12] Loeb, L., *J. Med. Research*, 1903–04, x, 407.
[13] Fleisher, M. S., and Loeb, L., *J. Biol. Chem.*, 1915, xxi, 477.

THE UNSATURATED FATTY ACIDS OF EGG LECITHIN.

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The work on liver lecithins by Levene and Ingvaldsen[1] and by Levene and Simms,[2] has shown the existence of lecithins which are distinguished from one another by the nature of their unsaturated fatty acids. There were isolated from liver lecithin, two unsaturated fatty acids, oleic and arachidonic, as well as two saturated acids, palmitic and stearic. Hence on the basis of the present findings it is permissible to accept the existence in the liver of four lecithins. Further work may lead to the discovery of an even greater number.

The work on the liver lecithins suggested the need of a reinvestigation of the lecithins of other organs with a view of finding in them also lecithins containing acids of a higher order of unsaturation than that of oleic acid.

The present communication contains the report of the work on egg lecithin. This "lecithin" also was found to contain more than one unsaturated fatty acid. Three acids were isolated; namely, oleic, linolic, and arachidonic. Oleic acid was identified by its iodine number and by its hydrogenation product, stearic acid. Linoleic acid was isolated in small quantities only and was identified as its tetrabromide. Arachidonic acid was identified by its octabromide and by its hydrogenation product, arachidic acid.

Comparing the mixed lecithins of liver and those of the egg yolk, one is struck by the difference in the proportions of the individual forms in them. The liver lecithin contains a very large proportion of the forms with highly unsaturated fatty acids, whereas in the egg yolk the proportion of the latter is small.

[1] Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 359.

[2] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1921, xlviii, 185.

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Thus, the iodine numbers of the cadmium chloride salts from liver lecithin varied between 59 and 84, whereas those from egg lecithins varied between 30 and 54.

It is noteworthy that individual samples of egg "lecithin" differed considerably from one another. An effort is being made to develop a method for fractionation of individual lecithins.

<div align="center">EXPERIMENTAL.</div>

A. Isolation of the Unsaturated Acids.

The source of the unsaturated acids was "lecithin cadmium chloride" free from amino-containing impurities. These cadmium chloride salts were prepared from both the acetone and ether extracts of egg powder and were purified by the methods described in previous papers.[3] Decided variation was observed among individual samples obtained either from different lots of egg powder or by different methods of extraction. However, it was not possible to establish a definite relationship between the composition of the material and these variable factors. The fatty acids were liberated by hydrolyzing the cadmium chloride salt for 6 hours with a 10 per cent solution of hydrochloric acid. On cooling, the mixed fatty acids separated as a solid cake. They were then dissolved in ether and thoroughly washed with water. The iodine numbers (method of Wijs) of several such whole fatty acids, are given below.

These acids were derived from salts which were obtained from different lots of egg powder, as well as by varying the methods of extraction.

No. 123 (from an "acetone extract"). 0.3114 gm. substance absorbed 0.2839 gm. iodine corresponding to an iodine number of 91.

No. 117 (from an "ethereal extract"). 0.3465 gm. substance absorbed 0.2839 gm. iodine corresponding to an iodine number of 82.

No. 301 (from an "ethereal extract"). 0.2798 gm. substance absorbed 0.2102 gm. iodine corresponding to an iodine number of 75.

After drying the ethereal solution and concentrating the solvent, a crude separation of the saturated from the unsaturated acids was effected by crystallization from acetone. The mother liquor

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[3] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xlvi, 195.

contains all the unsaturated, with a slight admixture of saturated acids. A more complete separation was accomplished through the insolubility of the barium and lead salts of the saturated acids in ether.

B. *Separation of Acids of Varying Degrees of Unsaturation.*

Two methods were used in an attempt to separate the constituent acids. The first procedure was based upon the greater solubility of the barium salts of the more highly unsaturated fatty acids in a benzene-alcohol mixture.[4] For this purpose $Ba(OH)_2$ was added to the mixed acids in a methyl alcoholic solution, until the solution was just alkaline to phenolphthalein. The mother liquor was decanted from the gummy precipitate, and concentrated to dryness under diminished pressure. The residue, combined with the original precipitate, was dissolved in a mixture of warm benzene and 5 per cent by volume of 95 per cent ethyl alcohol. To this, after cooling, an additional equal volume of ether was added. On standing over night in the ice box, barium oleate, together with the barium salts of any unsaturated acids present, was precipitated and was easily removed by filtration.

C. *Purification of Oleic Acid.*

The precipitated barium salts were decomposed with hydrochloric acid, and the liberated acids converted into their lead salts. The solubility of the lead salts of the unsaturated acids in cold ether, permitted the removal by filtration of the last traces of saturated acids. The unsaturated acid, obtained from its lead salt by treatment with hydrochloric acid, was a light yellow liquid which had an iodine value of 89, corresponding to oleic acid.

No. 307. 0.2578 gm. substance absorbed 0.2283 gm. iodine.
$C_{18}H_{34}O_2$. Calculated. Iodine value 90.
                Found.   "   "   89.

After hydrogenation by Paal's method the resulting saturated acid (No. 307) was analyzed and determinations of the molecular weight and melting point were made.

[4] Farnsteiner, K., *Z. Untersuch. Nahrungs- u. Genussmittel.*, 1899, ii, 1.

All samples of saturated fatty acids reported in this paper were dried by fusion on an electric hot-plate, and the material used for combustion was remelted under diminished pressure at the temperature of xylene vapor, until constant weight was obtained. The bromo acids were dried under diminished pressure at the temperature of boiling chloroform.

The melting points as recorded, are corrected, and were taken at such a rate that the time per degree rise in temperature was 6 seconds. The molecular weights were calculated by the titration of approximately 1 gm. of acid, dissolved in 10 cc. of toluene and 25 cc. of methyl alcohol (neutral to phenolphthalein) with 0.5 N NaOH, using phenolphthalein as an indicator.

No. 307. 0.1000 gm. substance: 0.1176 gm. H_2O and 0.2786 gm. CO_2.
          0.8465 "   "   required for neutralization 6.00 cc.
                                                              0.5 N NaOH.
$C_{18}H_{36}O_2$. Calculated. C 75.98, H 12.72. (Molecular weight = 284.
                                                Melting point = 70–71°C.)
          Found.   C 75.97, H 13.14. (Molecular weight = 282.
                                                Melting point = 71°C.)

D. Acids of a Higher Degree of Unsaturation.

The barium salts soluble in the mixture of benzene-alcohol-ether, as described under Section B, were decomposed with hydrochloric acid. The liberated acid, a dark brown liquid, had an iodine value of 165.

0.2055 gm. substance absorbed 0.3392 gm. iodine.

This acid after reduction by Paal's method gave the following analytical data.

No. 309. 0.1008 gm. substance: 0.1192 gm. H_2O and 0.2832 gm. CO_2.
          1.7580 "   "   required for neutralization 11.70 cc.
                                                              0.5 N NaOH.
$C_{18}H_{36}O_2$. Calculated. C 75.98, H 12.72. (Molecular weight = 284.
                                                Melting point = 70–71°C.)
$C_{20}H_{40}O_2$. Calculated. C 76.95, H 12.81. (Molecular weight = 312.
                                                Melting point = 75–77°C.)
          Found.   C 76.61, H 13.21. (Molecular weight = 299.
                                                Melting point = 66–67°C.)

E. Fractionation of the Methyl Esters.

For the fractional distillation of the methyl esters the crude unsaturated acids were converted into their lead salts. The ether-soluble fraction was decomposed with hydrochloric acid and the free acids were esterified. These methyl esters were then saturated with hydrogen by Paal's method and again esterified. The recrystallized esters were distilled at a pressure of 3 mm. into four fractions, each of which was then saponified and the free acid purified by conversion through the lead salt. The molecular weights, melting points, and analyses of the respective acids are recorded below.

No. 312. 0.1002 gm. substance: 0.1162 gm. H_2O and 0.2750 gm. CO_2.
   1.0132 "   "   required for neutralization 7.18 cc
   • 0.5 N NaOH.

No. 314. 0.1012 gm. substance: 0.1182 gm. H_2O and 0.2820 gm. CO_2.
   1.0216 "   "   required for neutralization 7.22 cc
   0.5 N NaOH.

No. 315. 0.1000 gm. substance: 0.1144 gm. H_2O and 0.2762 gm. CO_2.

No. 316. 0.0970 gm. substance: 0.1172 gm. H_2O and 0.2698 gm. CO_2.
   0.7858 "   "   required for neutralization 5.15 cc.
   0.5 N NaOH.

$C_{18}H_{36}O_2$. Calculated. C 75.98, H 12.76. (Molecular weight = 284.
   Melting point = 70-71°C.)

$C_{20}H_{40}O_2$. Calculated. C 76.95, H 12.98. (Molecular weight = 312
   Melting point = 75-77°C.)

	No.	Boiling point of ester. Pressure 3 mm.	Analysis of acid.		Weight of ester.	Molecular weight of acid.	Melting point of acid
			C	H			
		°C.			gm.		°C.
Found.	312	178–182	74.88	12.98	3.5	282	70–71
"	314	175–182	75 98	13 07	5.3	283	70–71
"	315	177–185	75.31	12.80	4.6		62–63
"	316	184–195	77.03	13.30	2.5	308	75

F. Bromine Addition Products of the More Highly Unsaturated Acids.

The acids, whose barium salts were soluble in benzene-alcohol-ether, were dissolved in 10 parts of glacial acetic acid. To the cooled solution a 10 per cent solution of bromine in glacial ace-

tic acid was added very gradually. The bromination was accompanied by a characteristic color change and the appearance of a very finely divided amorphous precipitate. After standing over night the latter was separated by centrifugalization and extracted with warm ether until further extracts were colorless. On drying, the material darkened slightly. When heated in an open tube it browned gradually above 215°, and contracted at 250°. It neither melted nor did it decompose with gas evolution. In a closed tube, this material after recrystallization from glacial acetic acid, contracted at 250°. It darkened decidedly above this point, and sintered very definitely at 255°.

By the hydrolysis of 380 gm. of a lecithin cadmium chloride (No. 123) whose iodine value was 54, mixed acids were obtained which had an iodine value of 91. From these, 60 gm. of acids, whose barium salts were soluble in a benzene-alcohol mixture, were isolated. These acids on bromination yielded 4.5 gm. of an octabromide insoluble in ether, but very slightly soluble in hot glacial acetic acid.

Analyses of two products obtained in this manner are given below:

No. 336.   0.0932 gm. substance: 0.3000 gm. H_2O and 0.0864 gm. CO_2.
          0.2068   "   "   0.3304   "   AgBr.
No. 304.   0.1024 gm. substance: 0.0326 gm. H_2O and 0.0942 gm. CO_2.
No. 362.   0.1846   "   "   0.2920   "   AgBr.
$C_{20}H_{32}O_2Br_8$. Calculated.   C 25.43, H 3.42, Br 67.72.
No. 336.   Found.   " 25.28, "   3.60, "   67.99.
No. 304.   "   " 25.08, "   3.56, "   67.32.

The bromination liquor, after the removal of the octabromide was concentrated to dryness under diminished pressure. The residual syrup was dissolved in ether and washed with sodium thiosulfate. From the concentrated ethereal solution, aggregates of spear-like needles were deposited. From the bromination of the acids of No. 123, details of which were given above, about 8 gm. of this crude material were obtained. This material was readily soluble in ether and absolute alcohol, but was insoluble in gasoline. After repeated recrystallization from various solvents, two samples, obtained respectively from an "ether" and an "acetone" extract, gave the following analyses, molecular weights, and melting points.

No. 355.   0.1034 gm. substance: 0.0512 gm. H_2O and 0.1368 gm. CO_2.
 0.2012   "   "   used for Carius determination: 0.2596 gm. AgBr.
 0.8401   "   "   requires for neutralization 13.70 cc. 0.1 N NaOH, corresponding to a molecular weight of 617.
In an open tube it softened at 115° and melted at 117–119°C.

No. 307.   0.1012 gm. substance: 0.0476 gm. H_2O and 0.1340 gm. CO_2.
 0.2010   "   "   0.2526 " AgBr.
 0.5412   "   "   required for neutralization 9.00 cc. 0.1 N NaOH, corresponding to a molecular weight of 601.
 Melted in an open tube at 116–117°C.

$C_{18}H_{32}O_2Br_4$. Calculated. C 36.01, H 5.38, Br 53.28. (Molecular weight = 600.)

No. 355.   Found.   C 36.08, H 5.54, Br 54.22.   (Molecular weight 617. Melting point = 115–116°C.)

No. 307.   Found.   C. 36.10, H 5.22, Br 53.48. (Molecular weight = 601. Melting point = 115–116°C.)

Tetrabromostearic acid was prepared by brominating the acids of cottonseed oil. This material when heated, contracted at 114° and melted at 115–116°C. When mixed with these tetrabromo acids from lecithin, no depression of its melting point was apparent.

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